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Pigment biosynthesis in Serratia marcescens

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PIGMENT BIOSYNTHESIS IN *SERRATIA MARCESCENS*

by

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INTRODUCTION

The microorganism Serratia marcescens characteristically produces a blood-red pigment which has been shown by a number of investigators to consist of several orange, red, and blue fractions (5, 31, 35, e.g.). The major fraction, called prodigiosin, has recently been shown, on the basis of n.m.r. data (29) and by synthesis (18), to have structure I (Figure 1). Norprodigiosin, the orange pigment produced by an orange mutant of S. marcescens, strain OF, has been shown to have structure II (Figure 1) by its conversion to prodigiosin upon treatment with diazomethane (36).

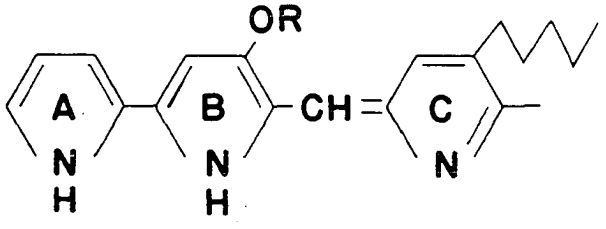

Figure 1. I Prodigiosin R = CH_3
II Norprodigiosin R = H

Similar pigments in species of the genus Streptomyces have been reported (7, 16, 24, 27), one of which, isolated from S. longisporus ruber, differs from prodigiosin only in the nature of substitution on pyrrole ring C (27, 30).

Pure prodigiosin has been reported to have strong anti-

biotic properties (8) and may have conferred a selective advantage to the organism; otherwise, the function of prodigiosin in Serratia is still unclear. Pigment production begins and reaches a maximum after cultures have passed the logarithmic phase of growth (32); cultures grown at 38° C do not produce pigment, but develop the same maximum viable count as cultures grown at 27° C, the optimum temperature for pigment production (34). The pigment has been shown to be associated with the cell envelope (17), but does not appear to confer any gross structural alteration upon pigmented cells compared to non-pigmented cells.*

An early proposal of a tripyrrylmethene structure for prodigiosin (38) stimulated many investigations on its biosynthesis, because of its possible relationship to a postulated tripyrrylmethane intermediate in the biosynthesis of porphyrins (25). The influences of such factors as pH, temperature, aeration, carbon and nitrogen sources, and metal ions on the production of pigment have been studied extensively (3, 12, 26, 34, e.g.), but investigations of this type have yielded little direct information on the pathway of prodigiosin biosynthesis.

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*R. P. Williams, Baylor University College of Medicine, Houston, Texas. Biosynthesis of prodigiosin and of pyrroles. Private communication. 1961.

More direct information has been gained through the use of isotopic tracers. The nitrogen and alpha-carbon atoms of labeled glycine are incorporated into prodigiosin, whereas the carboxyl-carbon is not (13). L-Proline is incorporated about two and a half times more efficiently than glycine, where the reverse is true in the case of porphyrins; furthermore, 5-aminolevulinic acid, a known precursor of the pyrrole rings of porphyrins, is apparently not incorporated (15). These findings, together with the proof of a pyrryldipyrrylmethene structure for prodigiosin, have cast doubt on the interrelationship of prodigiosin biosynthesis and porphyrin biosynthesis. Thus the possibility of finding an alternative pathway of pyrrole biosynthesis is presented by studies on Serratia marcescens.

One reaction in the biosynthesis of prodigiosin has been established through the use of the classical method of mutant analysis. The spontaneous occurrence of pink, orange, and white mutants in cultures of S. marcescens was shown by Labrum and Bunting (14); the frequency of such mutants can be increased under the influence of ultraviolet or gamma irradiation (14, 34). Rizki (19) and Williams and Green (33, 34) have demonstrated that several of these mutants are able to feed colorless diffusible substances to other mutants, enabling them to produce pigment. Santer and Vogel (23) isolated from a white mutant, S. marcescens strain 9-3-3, a

colorless pyrrole-containing compound which could be formed labeled from glycine-2-C^{14} and subsequently could give rise to labeled prodigiosin in another white mutant, strain W-1. This feeding-factor, later shown by Wasserman et al. (29) and by Rapoport and Holden (18) to be 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (III, Figure 2), condenses with pure synthetic 2-methyl-3-n-amyl-pyrrole (IV, Figure 2) in methanolic hydrochloric acid to form prodigiosin. Strain 9-3-3 produces prodigiosin when grown in the presence of vapors of synthetic methylamylpyrrole, and also produces pigment (presumably prodigiosin) when fed a volatile pyrrole (presumably methylamylpyrrole) by strain W-1 (28). These findings led Wasserman et al. (28) to propose the reaction shown in Figure 2 as a step in the biosynthesis of prodigiosin.

Except for this reaction and the data on incorporation of certain amino acids, little is known about prodigiosin biosynthesis. The work described in this thesis is an attempt to learn more about the biosynthesis of prodigiosin using the established method of mutant analysis.

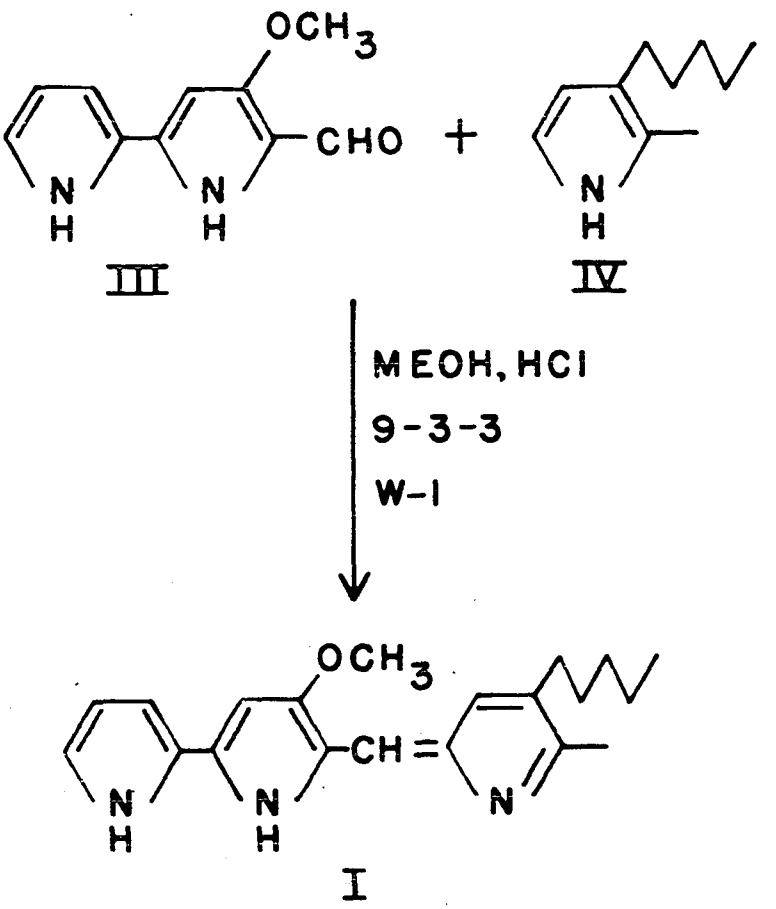


Figure 2. Known reaction in the biosynthesis of prodigiosin, I, in the 9-3-3 and W-1 strains of Serratia marcescens (28)

EXPERIMENTAL

Materials

Organisms

The microorganisms used in this study were supplied by Dr. Robert P. Williams of Baylor University College of Medicine, and have been previously described by Williams and Green (33, 34). The parent strain, Serratia marcescens strain Nima, produces a red pigment identified as prodigiosin. The two mutant strains, obtained by irradiation of strain Nima, are S. marcescens strain OF, which produces the orange pigment norprodigiosin (36), and S. marcescens strain WCF, which produces no pigment in pure culture but produces large amounts of red pigment when fed by strain OF (33). This red pigment, which will be designated fed-white pigment, has also been identified as prodigiosin in this investigation.

Media

Williams' medium. Williams' medium (35) consists of: yeast extract, 0.1 per cent; enzymatic casein hydrolyzate, 0.2 per cent; glycerol, 1.0 per cent; ammonium citrate, 0.5 per cent; dipotassium phosphate, 1.0 per cent; sodium chloride, 0.5 per cent; magnesium sulfate, 0.05 per cent; and ferric ammonium citrate, 0.005 per cent; made up in deionized water. The pH of the medium was checked and was always

7.0 ± 0.1.

Harned's medium Harned's medium (12) consists of: D-mannitol, 2.0 per cent; Bacto-Neopeptone, 1.0 per cent; and magnesium sulfate-heptahydrate, 0.125 per cent; made up in 40 per cent tap, 60 per cent deionized water (these proportions of tap water and deionized water were used because of the high mineral content in Ames tap water). The final pH was adjusted to 5.0 with 3N hydrochloric acid, unless otherwise noted.

Solid media contain, in addition to the above, 2.0 per cent agar for surface cultures and 1.2 per cent agar for stock slants.

Both media were usually made up in concentrations five times those described above, and diluted when needed.

Reagents and instruments

Reagents were CP or Analytical grade unless otherwise stated. Solvents for chromatography or counter-current distribution were routinely redistilled before use. The diatomaceous earth used for column chromatography was prepared from "Hy Flo Super Cel" (Johns-Mansville) according to the method described by Worthington (36). Diazomethane was prepared from "Diazald" (N-methyl-N-nitroso-p-toluenesulfonamide) by a method furnished by the Aldrich Chemical Company, Inc. Authentic 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde was

generously provided by Dr. Harry H. Wasserman, of Yale University.

Infrared spectra were obtained on a Perkin Elmer Model 21 Double Beam Infrared Spectrophotometer. Ultraviolet and visible spectra were obtained on a Cary Model 14 Recording Spectrophotometer or a Beckman Model DU Spectrophotometer. Absorbance readings at a given wavelength in the visible in the counter-current distribution experiments were made using a Bausch and Lomb Spectronic 20 Colorimeter instead of the Beckman Model DU Spectrophotometer. Measurements of pH were made on a Beckman Model G pH Meter. Melting points were determined on a melting point block.

Carbon, hydrogen, nitrogen, and chlorine analyses were conducted by the Ing. A. Schoeller Microanalytical Laboratory.

General Methods

Cultures

Stock cultures Williams' agar stock slants (1.2 per cent agar) were inoculated every six months and stored in a refrigerator at 8° C (4).

Large scale surface cultures Cells of Nima or OF were harvested from 5 day, 27° C Williams' agar cultures in 13 1/2 x 8 3/4 x 1 1/2 in. Pyrex baking dishes as described by Burgus (4). Cells from mixed cultures of OF and WCF were obtained similarly except that the inoculum for the plates was built

up in the following manner: a 250 ml. portion of Williams' broth in a 500 ml. prescription bottle was inoculated with one loop each of a 10 ml., 24 hr., 27° C Williams' broth culture of OF and a similar 10 ml. culture of WCF. The mixed OF-WCF broth culture was incubated for 24 hr. at 27° C, after which each Pyrex plate was inoculated with 20 ml. of this inoculum.

Harvested cells were lyophilized and stored in a deep-freeze until needed. Usually 20 plates were inoculated at one time.

Shake cultures As one means of obtaining small batches of broth cultures for pyrrole and feeding-factor studies, sterile, cotton-stoppered, 500 ml. Erlenmeyer flasks containing 100 ml. of Harned's broth were inoculated with 1 ml. of an 18 hr., 24° C, 10 ml. Harned's broth culture, which had been inoculated with a needle from a stock slant of the strain being investigated. The flasks were covered with foil and shaken at 24° C at a stroke length of 1.0 on a Burrel Model BB Wrist Action Shaker.

Thin-layer broth cultures Shake cultures were found to be too variable in total levels of pigments, pyrroles, and feeding-factor (possibly because of uneven aeration on the shaker) for precise determination of these factors from one run to the next. Better results were obtained using thin-layer broth cultures grown in Fernbach flasks. Sterile,

cotton-stoppered, 2800 ml. Fernbach flasks containing 100 ml. of Harned's broth were inoculated in a standard manner with 1.0 ml. of an 18 hr., 24° C, 10 ml. Harned's broth culture, which had been inoculated with a needle from a stock slant. The flasks were incubated, without shaking, at 24 ± 2° C unless otherwise stated.

Preparation of cell-free broth

To obtain cell-free broth for pyrrole and feeding-factor determinations, and cells for pigment determinations from shake cultures and thin-layer cultures, the contents of the flask (100 ml.) were poured, without rinsing, into a 250 ml. glass centrifuge bottle and centrifuged at 1800 x G.(2500 rpm) for 30 min. in an International Centrifuge, Size No. 2, Head No. 239. The supernatant was then carefully decanted and filtered using a sterile Seitz filtering apparatus prepared as follows: To a 500 ml. suction flask, plugged with cotton in the side arm, was attached a Size No. 6 Seitz funnel containing a Type S-1 asbestos filter sheet; the area around the funnel stem and the mouth of the flask was covered with aluminum foil and the entire filtering apparatus was sterilized by autoclaving. After filtration, the supernatant was transferred aseptically into a sterile, cotton-stoppered, 125 ml. Erlenmeyer flask and stored in a refrigerator.

For pigment determinations, the remainder of the cells

were rinsed from the culture flask with deionized water, the suspension was added to the cell residue from the first centrifugation, and the combined mixture was again centrifuged for 30 min. at 1800 x G. The supernatant was then carefully decanted and the cells were extracted for pigment determination.

Determination of pigments

The determination of pigment in a sample of cells was carried out essentially by the method of Harned (12). To the cell residue from a 100 ml. broth culture was added 40 ml. of 0.1 N methanolic hydrochloric acid (8.6 ml. of concentrated hydrochloric acid brought to 1 L. with absolute methanol). The suspension was thoroughly mixed and centrifuged at 1800 x G. for 20 min. The supernatant was then decanted into a 100 ml. volumetric flask, another 40 ml. of 0.1 N methanolic hydrochloric acid was added to the cell residue, and the suspension mixed and centrifuged as before. Usually, a third washing extracted no more pigment from the colorless cells; in some cases, only one washing was required to extract all of the pigment. The supernatants were combined, filtered through a fine fritted glass filter, and brought to 100 ml. with 0.1 N methanolic hydrochloric acid. An aliquot of this solution was diluted with 0.1 N methanolic hydrochloric acid to obtain an absorbance in the range of 0-0.5, at 525 mμ for

orange pigment, and at 537 mμ for red pigment. Readings were made vs. a 0.1 N methanolic hydrochloric acid blank.

For the red pigments, the concentration of the methanolic hydrochloric acid solution and subsequently the pigment content of the original sample was calculated assuming a value of 7.07×10^4 for ϵ max. at 537 mμ; Castro et al. (6) report ϵ max. at 540 mμ = 7.07×10^4 for prodigiosin hydrochloride in isopropanol. The molar extinction coefficient of the orange pigment is not known, so the same value of 7.07×10^4 was arbitrarily assumed for its molar extinction coefficient at its maximum of 525 mμ in methanolic hydrochloric acid. The use of this ϵ max. in this work is dependent on several assumptions; a unit of pigment will therefore be defined as 1 μmole of pigment calculated using the assumed extinction coefficients.

Determination of feeding-factor

Qualitative assay A simple qualitative assay for the presence of the orange mutant feeding-factor in broth samples, or in extracts, was essentially that described by Santer (22) for the detection of the feeding-factor produced by S. marcescens strain 9-3-3. To a petri plate containing 10 ml. of Williams' agar was added 5 ml. of seed agar, prepared by adding to 100 ml. of melted Williams' agar 5 ml. of an 18-24 hr. 24° C Williams' broth culture of the white mutant, WCF.

The plate was incubated for 24 hr. at 24° C and a penicillin assay disc saturated with sample was placed on the plate; in the case of samples containing organic solvents, the solvents were allowed to evaporate and the discs were saturated with water before they were placed on the plate. The appearance of a red coloration on or around the disc was taken to be a positive test. The intensity of the color could also serve for estimating the amount of the feeding-factor present.

Spots of the feeding-factor on paper chromatograms were developed in a similar manner. In this case, the chromatogram was laid on the surface of a 24 hr., 24° C, Williams' agar culture of the white mutant grown in a Pyrex baking dish. The spots containing feeding-factor usually showed red color within a few minutes, and were fully developed within an hour. The paper could then be removed from the plate, washed with water to remove excess cells, and dried in a 100° C oven for 30 min. to kill the bacteria, with the red spots remaining.

Quantitative assay Although the disc-plate assay would give an estimate of the amount of feeding-factor in a sample, a more quantitative assay was needed to determine the amounts of feeding-factor produced under different culture conditions and to follow its purification. To meet this need a procedure was used in which the amount of fed-white pigment produced by the white mutant in response to added feeding-factor was extracted and measured. Sterile, cotton-stoppered,

1 L. Roux flasks containing 50 ml. of Harned's broth were inoculated with 0.1 ml. of an 18 hr., 24° C, 10 ml. Williams' broth culture of the white mutant, WCF. The flasks were incubated in the dark for 24 hr. at 24° C, after which 10 ml. aliquots of sterile sample were added aseptically; samples were dissolved in Harned's broth, and were sterilized by filtration as described for the preparation of cell-free broth. Control flasks, to which 10 ml. portions of fresh Harned's broth were added, were included with each separate assay. After the addition of sample, the flasks were incubated for another 24 hr. at 24° C. The contents of the flasks were then centrifuged at 1800 x G. for 30 min. using an International, Size No. 2, Centrifuge, and the supernatant carefully decanted. The amount of red pigment in the cells was determined as described earlier in this section.

The dosage-response of the assay was checked by assaying 5, 10, 20, and 30 ml. samples of cell-free broth from a 48 hr., 24° C, shake culture of the orange mutant. Enough fresh Harned's broth was added to each sample to bring the total volume of added broth to 30 ml., and 30 ml. controls of fresh Harned's broth were included. A plot of units of pigment formed above control vs. dosage is shown in Figure 3. Since the amount of pigment formed was essentially linear in the normal range of concentrations of feeding-factor found in this investigation, 0-0.3 units per 10 ml. of added sample,

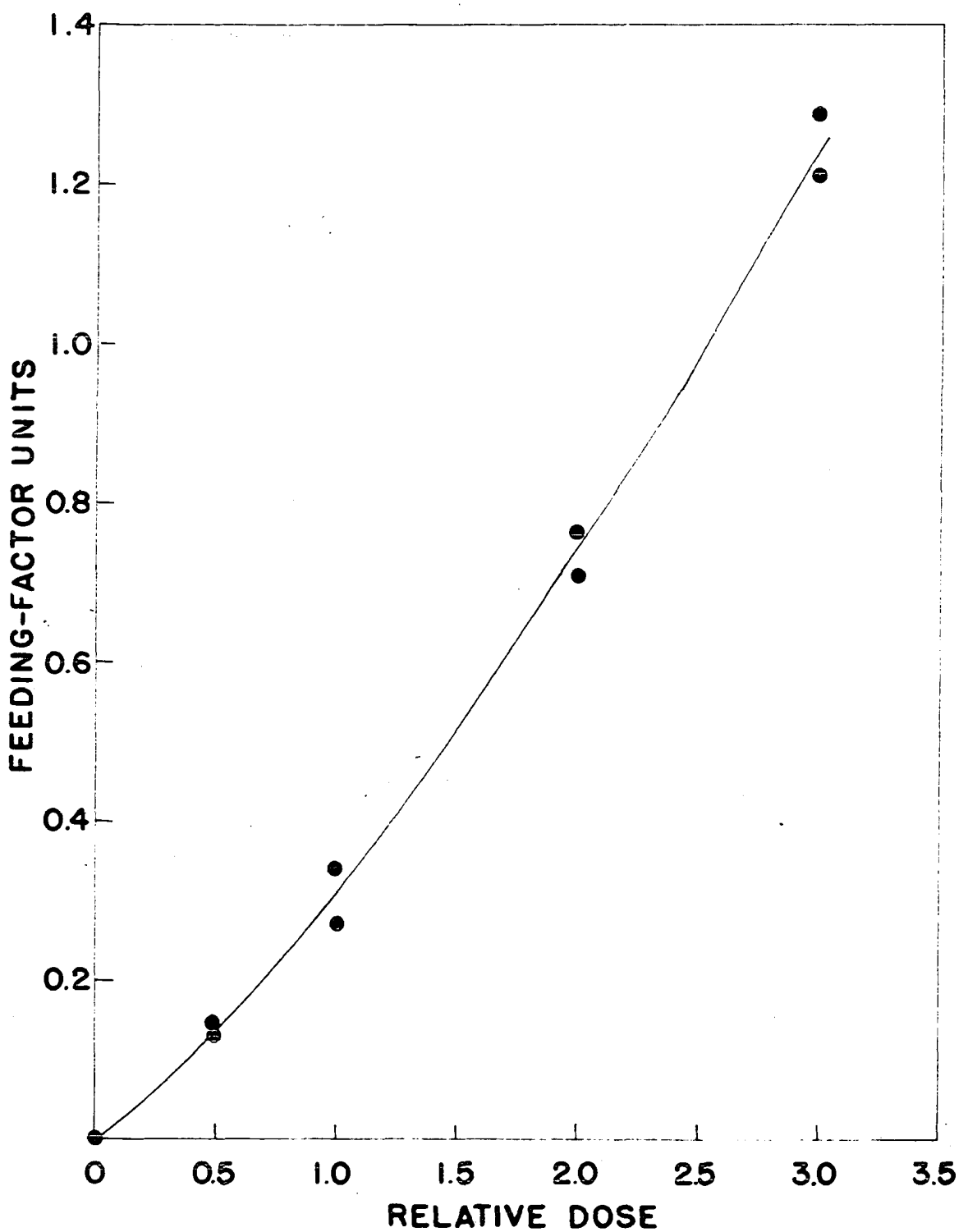


Figure 3. Dosage-response in feeding-factor assay

it was assumed that all of the feeding-factor present in the sample was incorporated into pigment; thus, after correction for control, 1 unit of feeding-factor activity will be defined as that amount of feeding-factor which gives rise to 1 unit of pigment.

Determination of pyrroles

Tests for pyrroles were based on the formation of dyes resulting from condensation of p-dimethylaminobenzaldehyde (p-DAB) with the pyrroles (the Ehrlich reaction). Pyrroles with free alpha-positions usually react with p-DAB in acid solution to give blue to red colors (9). The test is not specific for pyrroles; indole and some indole derivatives, for example, give positive tests. Also the color and intensity of the dye formed is dependent upon the type of substitution on the pyrrole nucleus. Santer (22) reports that the soluble precursor to prodigiosin produced by S. marcescens strain 9-3-3 gives a very weak Erhlich test; under different conditions either a red or a green color could be obtained. This finding was confirmed in our laboratory, and the same was found to be true for the feeding-factor produced by our orange mutant, strain OF.

Qualitative tests For spot tests and for spraying paper chromatograms, a 1 per cent methanolic solution of p-DAB was diluted 1 to 5 with 1 N methanolic hydrochloric

acid (86 ml. of concentrated hydrochloric acid diluted to 1 L. with absolute methanol).

Quantitative tests Determinations of the pyrroles in cell-free broth were carried out as follows: To 1.0 ml. of sample were added 1.0 ml. of 1 per cent p-DAB made up in 50 per cent aqueous methanol, and 5.0 ml. of 1 N methanolic hydrochloric acid (86 ml. of concentrated hydrochloric acid diluted to 1 L. with 75 per cent aqueous methanol); these proportions of methanol and water were chosen in order to avoid turbidity. The mixture was thoroughly shaken, allowed to stand at 25-30° C for one hour (the reaction was found to be complete after 45 min. at 25° C), and the absorbance of the solution at 550 mμ was read vs. an appropriate reagent blank. A control was always included in which 1.0 ml. of deionized water replaced the p-DAB. The control reading was subtracted from the sample reading and the pyrrole content was determined from a standard curve obtained using solutions of unsubstituted pyrrole (C_4H_5N). The useful region of the standard curve fell in the range of 0-60 μmoles of pyrrole per liter; if the sample did not produce a reading in this range, an appropriate dilution of the sample was made. One unit of pyrrole (Ehrlich-positive material) will be defined as that amount which corresponds to one μmole of pyrrole (C_4H_5N) under the conditions of this determination.

Unless stated otherwise, values reported for pyrrole

content in samples in Harned's broth include an average of 6 units per liter of Ehrlich-positive material which is present in fresh Harned's broth.

Determinations of pyrrole in non-aqueous samples were made using the reagents described for qualitative tests, with reference to an appropriate standard curve. In counter-current distribution experiments, the nature of the solvent systems sometimes required modification of the Ehrlich reagent in order to obtain single phase systems for absorbance readings. Solvent systems containing methyl cellosolve (2-methoxy-ethanol) formed a single phase more readily when ethanolic hydrochloric acid was used.

L-Trytophan does not interfere with the determination of pyrroles under the conditions described; a sample of 1 per cent aqueous tryptophan solution gave no absorbance at 550 mμ with the Ehrlich reagent used for cell-free broth. Indole, however, did interfere, but S. marcescens usually does not produce indole, as determined by Kovac's test (p-DAB in amyl alcohol) (2).

Counter-current distribution

Counter-current distribution experiments were carried out using a modification of the micro-distribution apparatus described by Bell et al. (1). The hand-operated, all-glass machine, constructed in the glass shop of Iowa State Univer-

sity, consists of one row of 60 tubes, each with a 1 ml. lower phase, 1 ml. upper phase capacity.

The first tube of the machine was charged with the sample dissolved in 1 ml. of each phase of the solvent system, the desired number of transfers were run (in a constant temperature room at 24° C), and the combined upper and lower phases were collected from each tube. When 20 transfers were run, it was convenient to run two samples at the same time, using tubes 0-20 for one distribution and tubes 30-50 for the second.

When pigments were distributed, a standard amount of acetone (usually 6.0 ml.) was added to each tube to insure the formation of a single phase. A drop of 3 N hydrochloric acid was added to each fraction to increase the absorbance and the absorbance was read at 540 mμ vs. an appropriate solvent blank.

When the contents of the tubes were to be analyzed for pyrroles, standard amounts of alcohols were used instead of acetone to obtain single phase systems, since acetone and pyrrole produced a brown color in acid solution. Methanol was used when samples were distributed in the butanol and methanol solvent systems, and ethanol was used with the methyl cellosolve solvent systems. In both cases the alcohol and the corresponding 2 N alcoholic hydrochloric acid were used in the proper proportions to obtain a final hydrochloric acid

concentration of 0.7 N (the final concentration in the test designed for cell-free broth). Color was developed in the even-numbered tubes by including a 1.0 ml. portion of 1 per cent p-DAB in the alcohol added. The odd-numbered tubes were used as controls. The tubes were allowed to stand for one hour at room temperature and the absorbances of the samples and controls were read vs. an appropriate blank at 550 mμ.

To find the approximate distribution coefficient K from the experimental plots, the expression

$$K = \frac{N}{n - N}$$

was used; where N = the number (not necessarily integral) of the peak tube, and n = the total number of transfers.

Paper chromatography

Whatman No. 1 paper was used throughout this investigation, and chromatograms were developed ascendingly in 15 cm. (diameter) x 46 cm. plate-glass covered jars at 24° C in the dark. When two-phase solvent systems were used, beakers of lower phase were placed in the jars.

Prodigiosin in Wild-Type and Fed-White Pigments

Since the major purpose of this investigation was to shed some light on the biosynthesis of prodigiosin, it was desirable to establish with certainty that the Nima and fed-white pigments were indeed prodigiosin. These pigments were there-

fore isolated and their properties compared in a number of ways with those of prodigiosin.

The same procedures were used throughout for extraction and purification of both the Nima and the fed-white pigments.

Extraction and preparation of salts

The extraction of the pigments, preparation of hydrochlorides, and conversion of the hydrochlorides to perchlorates were carried out according to the methods described by Wrede (37). To 50 g. of lyophilized cells were added 400 ml. of deionized water and 250 ml. of 10 per cent aqueous sodium hydroxide; the orange suspension was thoroughly mixed and allowed to stand for 3 hr. at room temperature. The suspension was then transferred to a separatory funnel and 500 ml. each of petroleum ether (b.p. 65-67° C) and 95 per cent ethanol were added with thorough mixing. After standing for 8 hr. at room temperature, the mixture had separated into two layers: a very turbid upper layer containing suspended particles and a dark orange-brown lower layer which was drawn off and discarded. The upper layer was shaken four times with 500 ml. portions of petroleum ether and the petroleum ether layers which separated were combined. The combined extracts were concentrated *in vacuo* at 40-50° C to about 250 ml., washed with three 500 ml. portions of deionized water, and dried with anhydrous sodium sulfate. After the sodium sulfate

had been removed by filtration, the dry petroleum ether solution was concentrated to 50 ml. and dry hydrogen chloride was bubbled through the solution for 20 min. The hydrochloride, which precipitated as a brick-red, amorphous solid, was collected and dried. The yield of hydrochloride of the fed-white pigment was about 7 mg. per g. of lyophilized cells; of the wild-type pigment, about 3 mg. per g. of lyophilized cells.

The perchlorate was prepared from the hydrochloride by dissolving 300 mg. of the hydrochloride in 25 ml. of warm 95 per cent ethanol. The solution was filtered to remove the small amount of undissolved material, and 5 per cent aqueous perchloric acid was added dropwise until the solution became turbid. The perchlorate crystallized in small, red needles, which appeared to sinter at 200° C and decompose at 215° C. After recrystallization from 95 per cent ethanol-5 per cent perchloric acid, the crystals appeared to sinter at 205° C and decompose at 222° C (uncorrected).

Counter-current distribution

The hydrochlorides and the perchlorates were distributed in petroleum ether (b.p. 65-67° C):methyl cellosolve:0.01 M phosphate buffer, pH 7.2 (4:3:1). The salts of both the wild-type and the fed-white pigments gave distribution curves identical to that of authentic prodigiosin, m.p. 150.2-152° C, received from Dr. Robert P. Williams, of Baylor University

College of Medicine. After 20 transfers, most of the material occurred in a peak with a K = 0.82, as shown in a typical plot in Figure 4.

None of the fractions of orange pigment from pure cultures of the orange mutant, OF, contained material with a K = 0.82 in this solvent system (36). It would therefore appear that the fed-white pigment hydrochloride contained very little admixed orange pigment.

Column chromatography

The pigments were further purified by chromatography of the free bases prepared from the perchlorates. The wild-type and the fed-white pigments were essentially identical in their behavior on columns, so a typical purification of the wild-type pigment will be described. About 125 mg. of the perchlorate was taken up in 100 ml. of 95 per cent ethanol and 10 per cent aqueous sodium hydroxide was added until the solution turned yellow-brown; usually about 0.5 ml. of base were required. To the ethanol solution was added 200 ml. of deionized water and the mixture was extracted with three 50 ml. portions of petroleum ether (b.p. 65-67° C). The combined petroleum ether extracts were washed with three 250 ml. portions of deionized water, filtered to remove a red-brown grease which occurred at the interface, and then concentrated *in vacuo* at 40-50° C to about 50 ml.

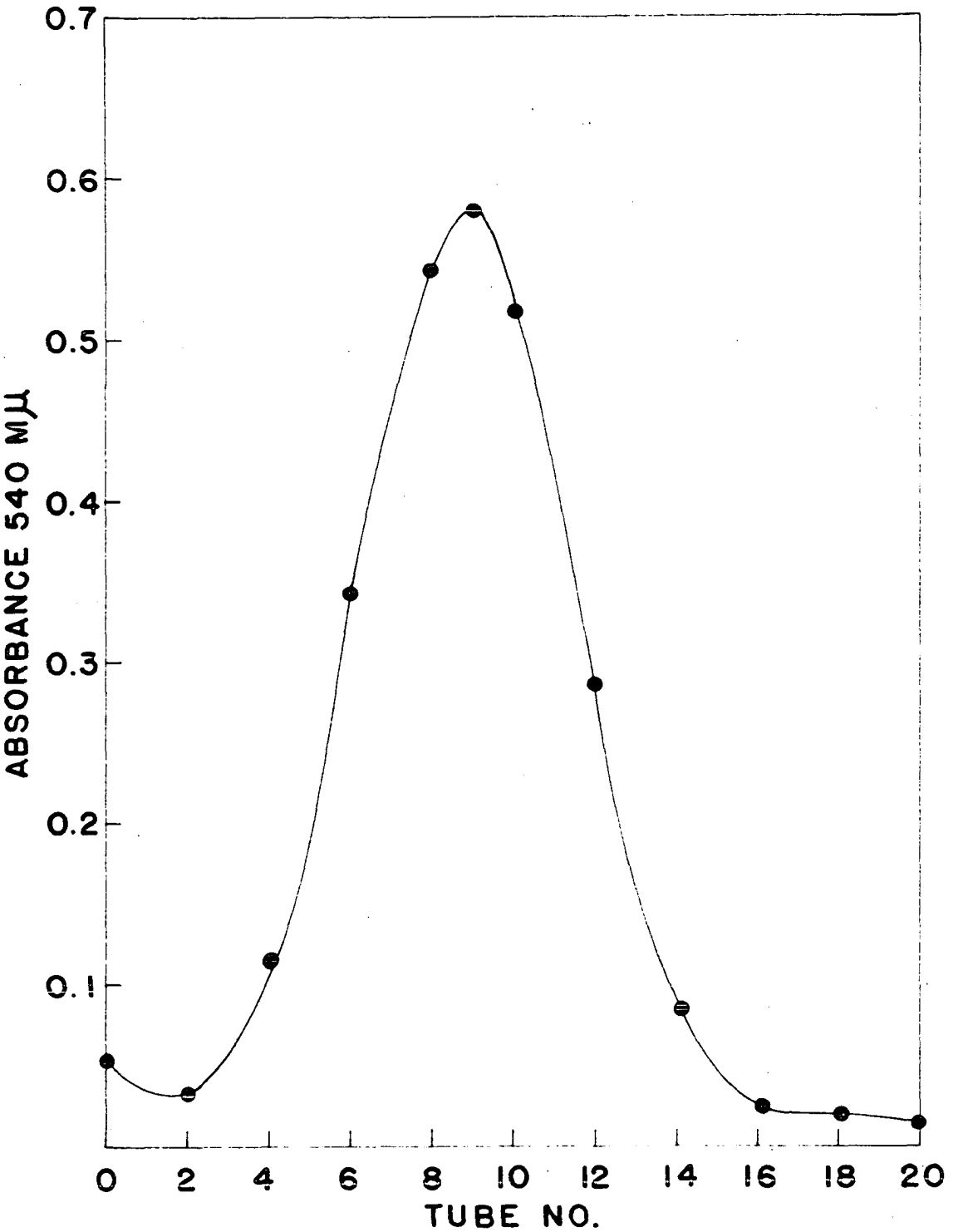


Figure 4. Counter-current distribution of perchlorate of fed-white pigment in petroleum ether:methyl-cellosolve:0.01 M phosphate buffer, pH 7.2 (4:3:1)

The concentrated petroleum ether extract was applied to a 4.0 cm. (diameter) x 30 cm. column of Hy Flo Super Cel prepared by pouring a slurry in petroleum ether. After development with about 50 ml. of petroleum ether, there were three main bands: (a) a red-orange band, moving with the solvent front; followed by (b) a red band, which eluted very slowly in petroleum ether; followed by (c) a dark magenta band, which remained at the top of the column. The red-orange band (a) was eluted with 100 ml. of petroleum ether as an orange eluate which was concentrated and applied to a second, smaller column of Hy Flo Super Cel. Upon development with petroleum ether, the appearance of the column was essentially the same as that described for the first column; i.e., a red-orange, a red, and a magenta band separated. The red-orange band was again eluted and re-chromatographed on a third column, with the same results.

After the red-orange band (a) had been eluted from the first column with petroleum ether, the column was developed with 2 per cent absolute ethanol in petroleum ether, whereupon the red band (b) was eluted as an orange eluate and the magenta band (c) remained on the column.

The eluates (a) and (b) behaved identically when re-chromatographed with 2 per cent ethanol in petroleum ether, so these fractions were combined for further purification. The solvent was removed <u>in vacuo</u> at 40-50° C; the residue

(about 70 mg.) was taken up in a few ml. of 2 per cent ethanol in petroleum ether and applied to a 2.5 cm. (diameter) x 30 cm. column of Hy Flo Super Cel, poured in a slurry of the developing solvent. Upon development with 2 per cent ethanol six bands separated: one red-orange band; two red bands; and three magenta bands. Four fractions were collected: the red-orange band, the combined red bands, and one of the magenta bands, in that order, were eluted with 2 per cent ethanol; and the two remaining magenta bands were eluted with absolute ethanol. These fractions will be designated red-orange, combined-red, magenta-1, and magenta-2, respectively. The yields were: red-orange, 57 mg.; combined-red, 3 mg.; magenta-1, 2 mg.; and magenta-2, 3 mg. Yields were comparable for the fed-white pigments.

Comparison of the purified pigments with prodigiosin

The ultraviolet and visible spectra of acidic ethanol solutions of the red-orange column fractions derived from the wild-type and the fed-white pigments are shown in Figure 5, and the infrared spectra (in KBr) in Figure 6. The ultraviolet, visible, and infrared spectra of the two pigments are essentially identical to those reported for prodigiosin by Castro et al. (5). The same fractions gave single peaks identical to authentic prodigiosin (Figure 4) when distributed in the petroleum ether-methyl cellosolve-phosphate buffer

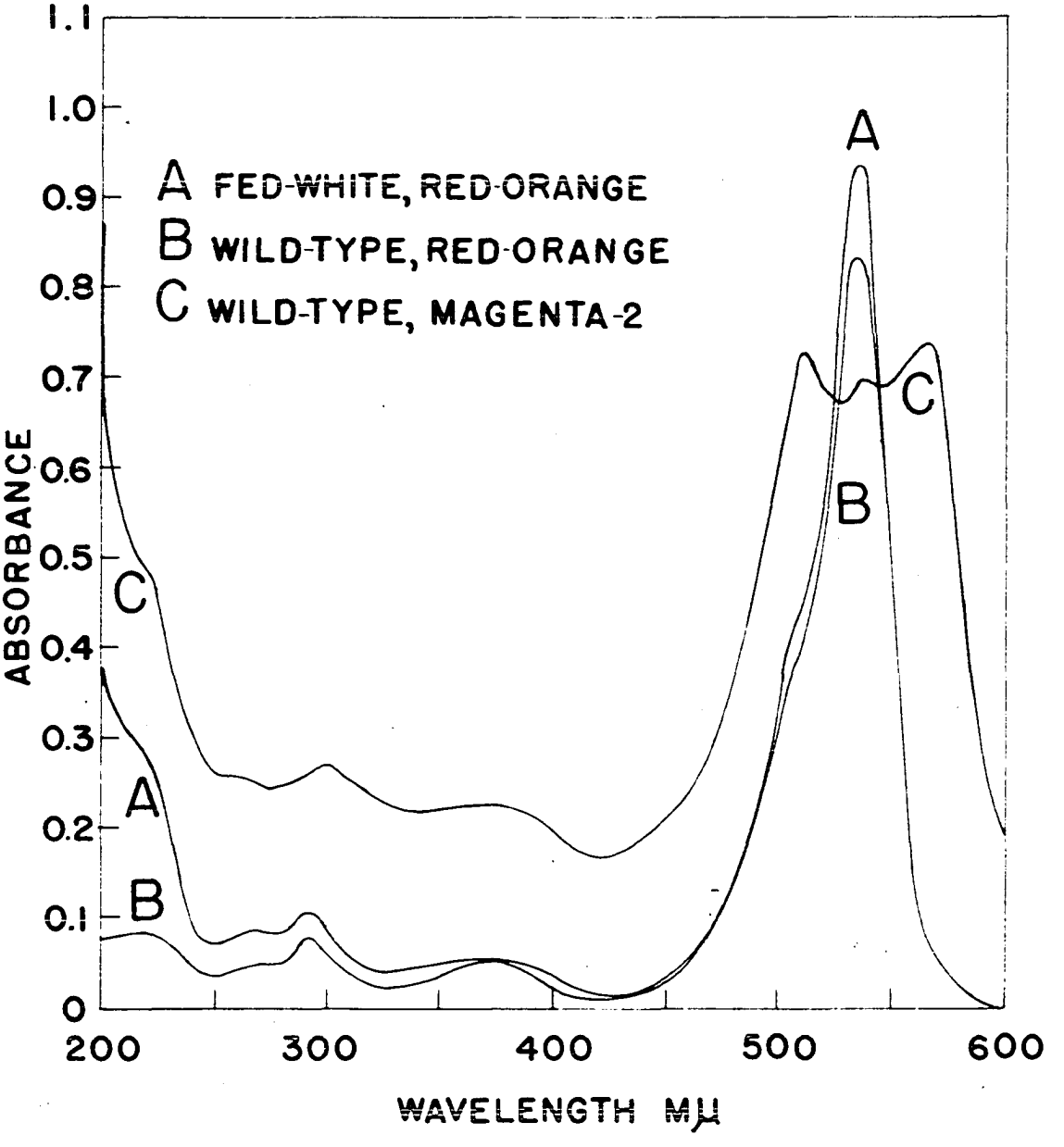
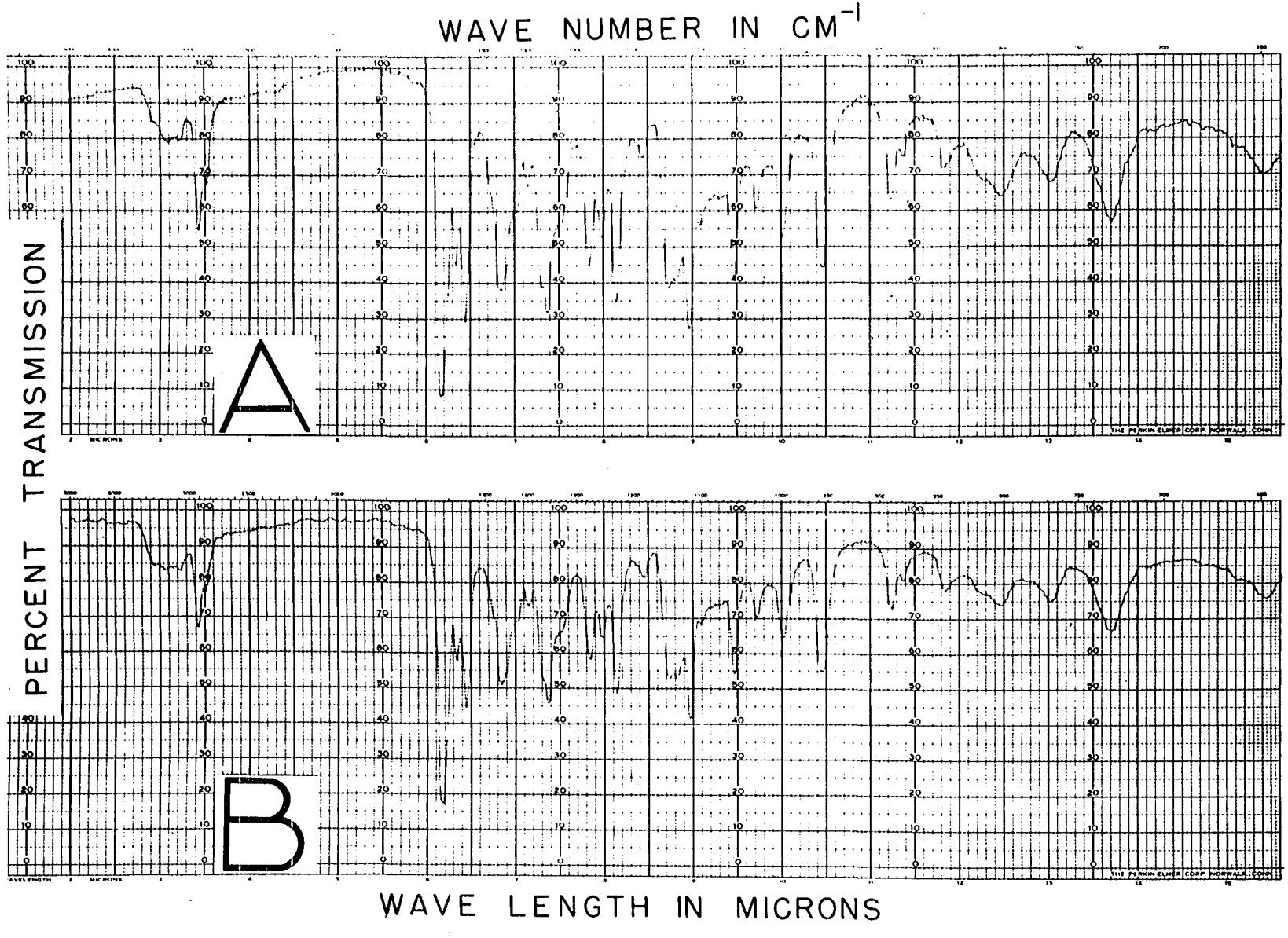


Figure 5. Spectra of prodigiosin column fractions in acidic ethanol (10 ml. ethanol solution + 1 ml. 0.1 N HCl)

Figure 6. Infrared spectra of A, wild-type red-orange fraction, and B, fed-white red-orange fraction, in KBr


WAVE NUMBER IN CM⁻¹
PERCENT TRANSMISSION
A
B
WAVE LENGTH IN MICRONS

solvent system.

The red-orange fractions were crystallized as perchlorates from 95 per cent ethanol with a 70 per cent yield. The perchlorates sintered at 205° C and decomposed at 222° C (uncorrected), as determined by the disappearance of birefringence.

Analyses Calculated for $C_{20}H_{26}N_3O_5Cl$: C, 56.67%; H, 6.18%; N, 9.91%; and Cl, 8.36%. Found for perchlorate from wild-type: C, 57.03%; H, 6.20%; N, 9.75%; and Cl, 8.38%. For perchlorate from fed-white: C, 56.36%; H, 6.31%; and N, 9.74%.

Properties of other column fractions

The combined-red fraction was identical to the red-orange fraction in counter-current distribution. The ultraviolet and visible spectra of acidic ethanol solutions of the two fractions were essentially identical, and the infrared spectra differed only in the relative intensities of certain peaks.

The magenta-1 and magenta-2 fractions, in contrast to the red-orange and combined-red fractions, were insoluble in petroleum ether; the magenta fractions were soluble in benzene, chloroform, and .2 per cent ethanol in petroleum ether. Counter-current distribution of the magenta-2 fraction in the petroleum ether-methyl cellosolve-phosphate buffer solvent system gave material with K = 0 and also with K = ∞, but only

a trace of material corresponding to prodigiosin (K = 0.82). The ultraviolet and visible spectrum of the magenta-2 fraction is different from the spectrum of prodigiosin, as shown in Figure 5, but resembles very closely the spectrum of a blue component of wild-type and fed-white pigment reported by Williams et al. (10, 11, 35). Re-chromatography of the magenta-2 fraction on Hy Flo Super Cel columns developed with 2 per cent ethanol gave several magenta bands.

Orange, red, and blue (or magenta) fractions of Serratia pigments have been reported by several investigators (5, 10, 11, 31, 35). The disproportionation of these fractions observed in this investigation suggests that the red-orange and the red fractions, which both yield prodigiosin, are in equilibrium in solution. Castro et al. (5) have suggested that prodigiosin exists mostly as the dimer in benzene solution. The orange and the red fractions might result from the association of prodigiosin. Another possibility is that prodigiosin can exist in interconvertable tautomeric forms, which differ slightly in solubility.

The magenta (or blue) fractions appear to result from an irreversible alteration of the orange or red fractions.

Cross-Feeding in Nima Strains

General observations

The presence of a factor in cultures of the orange mutant, OF, which enables the white mutant, WCF, to produce pigment had been previously established (33, 34). Moreover, this factor was presumed to have some stability outside of the cells of the orange mutant, since the white mutant was fed through an agar medium separating the two mutants. However, early attempts to detect the feeding-factor in cell-free broth or in extracts of the broth from 28° or 37° C, 48 hr. cultures of the orange mutant were unsuccessful. In these early experiments, samples were added to Harned's broth, which was then inoculated with the white mutant. No prodigiosin above control levels developed upon incubation of the white mutant cultures. The failure of this assay prompted further investigation of the cross-feeding relationships of the orange and white mutants.

The loss of an extremely volatile feeding-factor was ruled out by growing the two mutants in divided petri dishes. Partitioned petri dishes containing Williams' agar were seeded with OF and WCF such that the two mutants were separated by partitions; after 48 hr. of incubation at 27° C, there was the usual amount of orange pigment in the OF cultures, but no red pigment in the WCF cultures.

It was also considered that the failure to demonstrate the presence of the feeding-factor outside of mixed cultures (or cultures separated only by agar) might be due to some sort of mutual cross-feeding existing between the two mutants. The orange mutant requires nothing from the white mutant in order to produce orange pigment, but it was reasoned that orange pigment might result from a shunt pathway, and that the feeding-factor would not necessarily have to be incorporated into the orange pigment. The orange mutant might, however, be capable of converting to feeding-factor a precursor which it could not make, but which was provided by the white mutant; thus, the orange mutant would have to be grown in the presence of the white mutant in order to produce the feeding-factor. In order to establish the presence or absence of such a mutual cross-feeding relationship, the mutants were grown in a system in which there was a uni-directional diffusion of metabolites from one mutant to the other, according to a method described by Rizki (20).

The apparatus, built according to a design described by Roberts (21) for high-temperature chromatography, consisted of an 8 x 6 x 2 in. plate-glass covered, stainless steel chamber. A 2 1/2 in. strip of Whatman No. 1 paper, supported by glass rods, extended from a reservoir of Williams' broth at one end of the chamber to adsorbant cotton at the other end of the chamber. The rate of flow of the broth toward the

cotton was checked with dye and found to be 1-2 cm. per hour. The apparatus was sterilized by autoclaving.

Plates of Williams' agar were inoculated with loops of 24 hr., 27° C broth cultures of OF and WCF, respectively, such that growth occurred in areas about 1/2 cm. in diameter; the plates were incubated for 12 hr. at 27° C., after which small blocks (about 1 cm. square) were cut around the areas of growth and placed aseptically on the paper strip in the apparatus. Care was taken to avoid contamination of the paper with the mutants, since the motile organisms can move freely on wet paper. The system was incubated at 27° C.

When the blocks were placed about 2 cm. apart such that metabolites could diffuse only from the orange mutant to the white mutant, red pigment developed in the cells of the white mutant within 12 hr., increasing in intensity up to 72 hr., when the experiment was stopped. The orange mutant appeared to develop as much orange pigment under these conditions as it did on agar plates.

Detection of feeding-factor in cell-free broth

Having established that the feeding-factor was not volatile and that the cross-feeding was uni-directional, only two explanations for the failure of the assay remained: either the feeding-factor was unstable or the white mutant degraded it during the time between inoculation and differentiation of

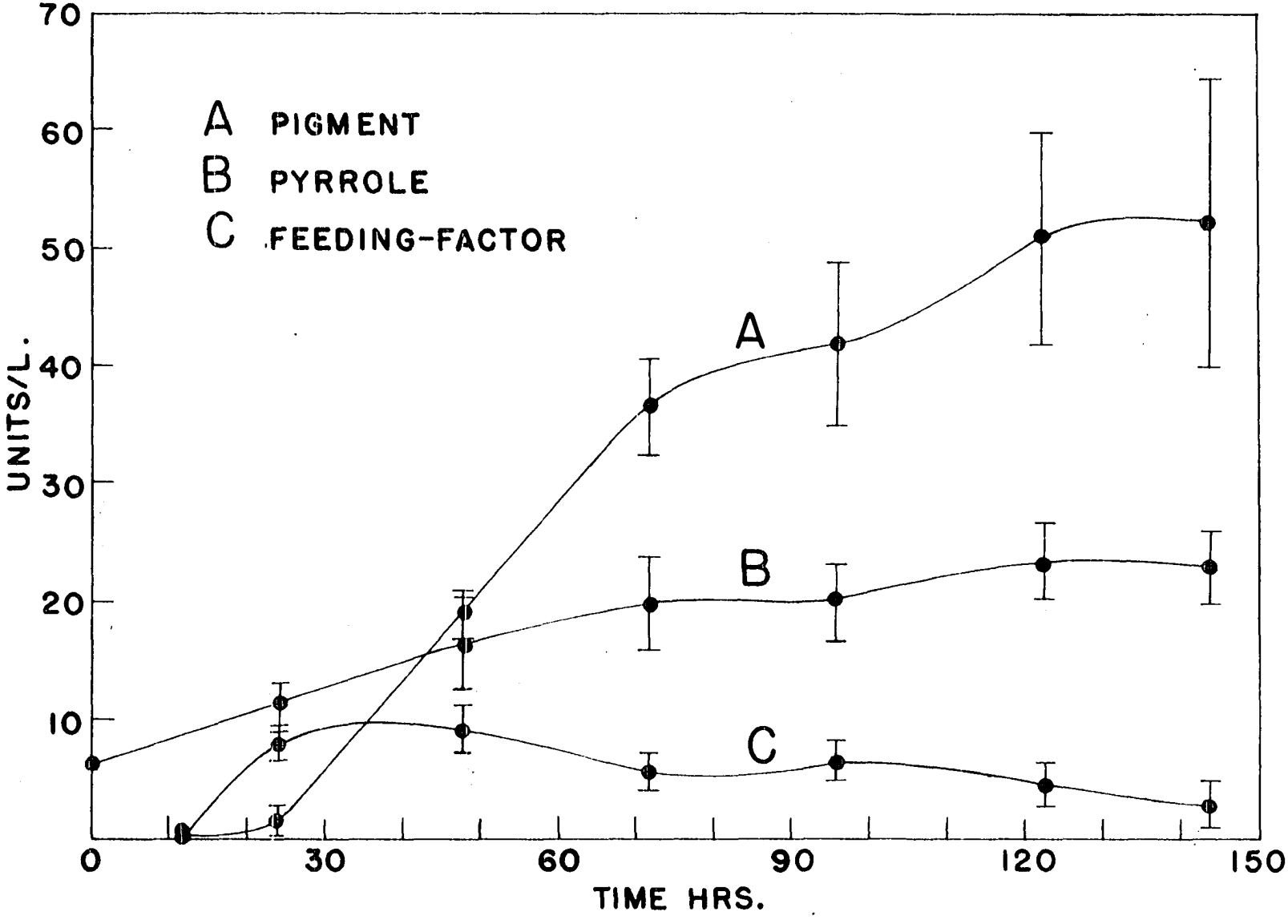
the culture to form pigment. In either case it should be possible to detect the feeding-factor by adding the fresh cell-free broth to a developed culture of the white mutant. It was found that the feeding-factor could indeed be detected under those conditions and the quantitative bio-assay for feeding-factor using the white mutant was developed as described under "General Methods" above.

Factors affecting production of feeding-factor

In order to isolate and characterize the feeding-factor, it was desirable to determine the optimum conditions for its production, and to learn more about its relationship to orange pigment and pyrroles in the orange mutant. The production of feeding-factor, orange pigment, and pyrroles as a function of time in thin-layer Harned's broth cultures of the orange mutant is illustrated in Figure 7. The shape of the curve for pigment production compares favorably with that reported for prodigiosin in Nima cultures grown under similar conditions (32). Pyrroles and feeding-factor are both produced before pigment production starts at a high rate; therefore, both could be involved in the synthesis of pigment. The decrease in total feeding-factor after 45-50 hr. could result from its instability, its incorporation into orange pigment, or both.

Since it was known that pigments are not produced at

Figure 7. Production of pigment, pyrrole, and feeding-factor by orange mutant vs. culture time (24° C); bars represent average deviation from the mean


A PIGMENT
B PYRROLE
C FEEDING-FACTOR
UNITS/L.
70
60
50
40
30
20
10
0
30
60
90
120
150
TIME HRS.
A
B
C

38° C, it was of interest to determine the effect of elevated temperature on the production of feeding-factor. If the feeding-factor were being incorporated into orange pigment and the temperature-dependent step in pigment biosynthesis occurred after the formation of feeding-factor, it might be possible to increase the yield of the feeding-factor by growing the orange mutant at the higher temperature. However, when thin-layer cultures of the orange mutant were grown for 48 hr. at 38° C, only 0.7 units of feeding-factor were found per liter of cell-free broth, as opposed to 9.3 units per liter in cell-free broth from similar 24° C cultures.

Santer has reported (22) that the initial pH of the medium has an effect on the production of the precursor to prodigiosin produced by strain 9-3-3; the optimum was pH 7.5 in a synthetic medium consisting of glycerol, ammonium citrate, amino acids, and salts. The pH optimum for the production of feeding-factor by the orange mutant, OF, was not determined in this investigation, but it was found in one experiment that more feeding-factor was produced in Harned's broth with an initial pH of 5.0 than with an initial pH of 7.5.

The total levels of feeding-factor were higher in shake cultures than in thin-layer cultures under the same conditions; 48 hr., 24° C shake cultures averaged 27 units of feeding-factor per liter, as opposed to 9.3 units per liter

in similar thin-layer cultures. Therefore 48 hr., 24° C shake cultures were used to obtain cell-free broth for experiments on isolation and characterization of the feeding-factor.

Isolation and Characterization of the Feeding-Factor

Extraction and purification

No feeding-factor activity could be detected in methanol extracts of cells from 42 hr., 24° C surface cultures of the orange mutant, so cell-free broth was used as a source for isolation of the compound. In early experiments on the extraction of feeding-factor from cell-free broth it was found that the presence of residual organic solvents interfered to some extent with the bio-assay. Results could be improved by including controls saturated with the solvent present in the sample, but the reproducibility of the assay was reduced. Since it seemed a logical assumption that the feeding-factor might contain a pyrrole group in view of the structure of prodigiosin, extraction of the cell-free broth was monitored using the Ehrlich test for pyrroles; solvents which appeared promising were then tested using the more complicated bio-assay. It was later found that the feeding-factor, although a pyrrole with a free alpha position, gave a very weak Ehrlich test; thus, the level of pyrrole in the test sample did not necessarily reflect the level of feeding-factor.

Also, some of the early extraction experiments were

carried out on cell-free broth samples which had been stored in a refrigerator for a few days. It was subsequently found that about 15 per cent of feeding-factor activity was lost on storing the cell-free broth in a refrigerator for 24 hr., and about 85 per cent was lost within 5 days. After it was established that the feeding-factor was unstable, only fresh samples were used.

Some of the solvents investigated, such as n-butanol, would remove most of the feeding-factor activity from the cell-free broth, but no activity could be recovered from the extract after the solvent had been removed *in vacuo* at 40-50° C. Extraction of the broth with three half-volume portions of diethyl ether removed about 40 per cent of the feeding-factor activity; about 25 per cent of the activity in the original broth could be recovered after removing the ether *in vacuo* at 30-35° C. In spite of this inefficiency, extraction with ether was chosen as a means of obtaining concentrated fractions of the feeding-factor under mild conditions. The average yield at this step was about 7-8 units of feeding-factor per liter of broth, based on the bio-assay.

Partial purification by paper chromatography The feeding-factor could be purified to some extent by elution of streaked chromatograms. The ether extract from 800 ml. of fresh cell-free broth was streaked on a 9 cm. line, 2 cm. from the bottom of a 10 cm. x 46 cm. strip of Whatman No. 1 paper,

and the paper was developed for 20 hr. at 24° C in n-butanol: 1 N aqueous ammonia (1:1) or n-propanol:1 N ammonia (4:1). After drying the paper at room temperature away from direct sunlight, a lengthwise 1 cm. strip was cut from the center for assay on a plate of the white mutant. After the spot containing the feeding-factor had developed on the assay strip, the Rf was determined and an area including this Rf was cut from the rest of the chromatogram; usually a 4 cm. strip was taken. The strip containing the feeding-factor was then cut into pieces about 2-3 mm. square and eluted with five 20 ml. portions of absolute methanol. An estimated yield of 0.3-0.4 units of feeding-factor per liter of broth in this step was calculated from the absorbance of the methanol eluate at 363 mμ, using an assumed ϵ max. = 3.5×10^4, the value reported by Santer and Vogel (23) for 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde at 363 mμ, in ethanol. The eluted paper was found to have no feeding-factor activity when assayed on a plate of the white mutant.

Chemical and physical properties of the feeding-factor

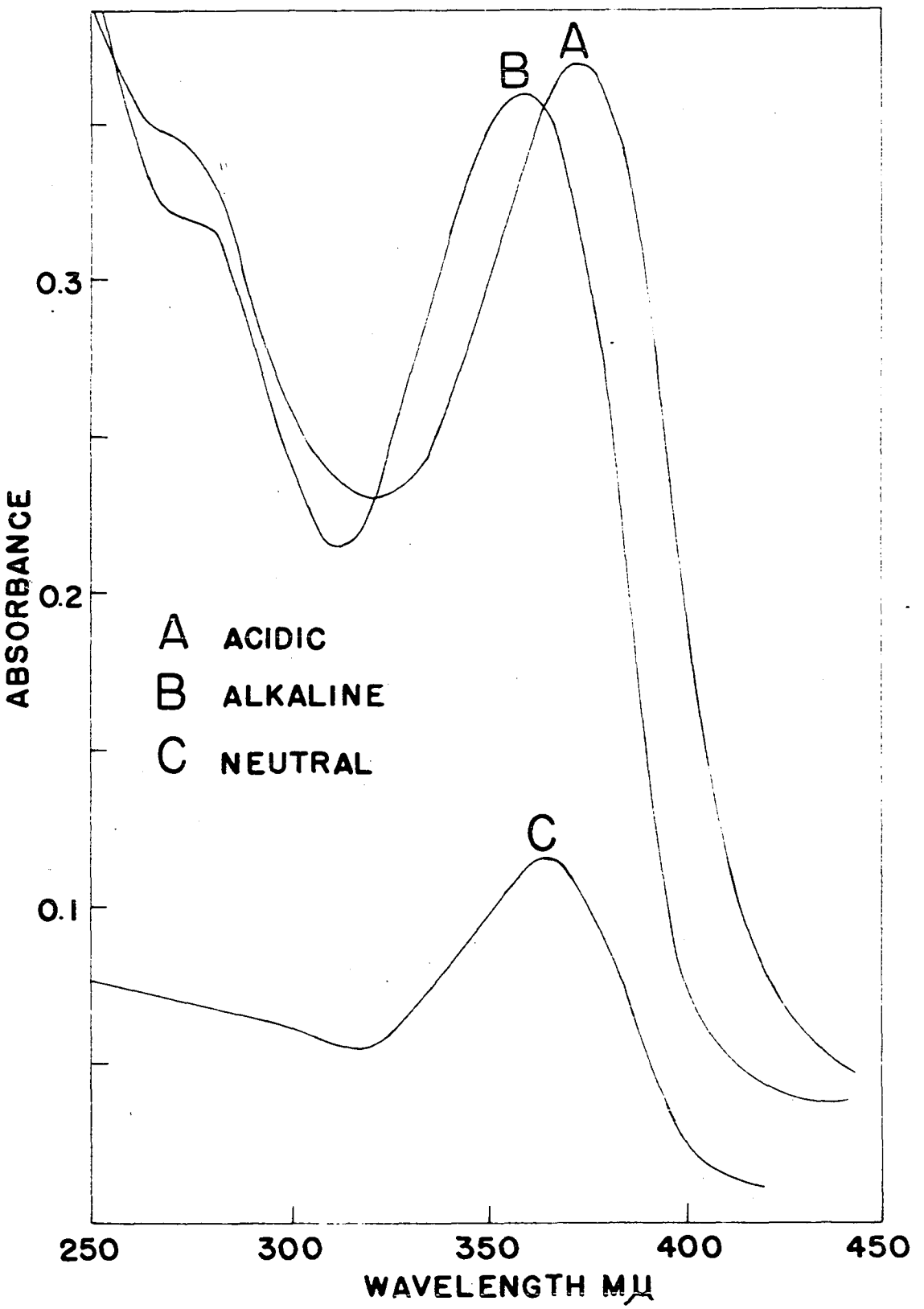
Spectral properties The ultraviolet spectrum of the methanol eluate of the feeding-factor from paper chromatograms was determined under neutral, acid, and alkaline conditions. The spectrum under neutral conditions was obtained using the untreated methanol eluate; acid and alkaline conditions were

obtained by adding 1 ml. of 0.1 N hydrochloric acid and 1 ml. of 0.1 N sodium hydroxide, respectively, to 10 ml. of the methanol eluate, concentrated to one fourth its original volume in vacuo at 35° C. The results are shown in Figure 8.

Under neutral, acid, and alkaline conditions the major absorption maxima are 365, 370, and 358 mμ, respectively; the details at lower wavelengths are somewhat obscured, probably because of impurities, but a shoulder at about 275 mμ is apparent in the spectra of the more concentrated acid and alkaline solutions. Santer and Vogel (23) have reported maxima at 363 mμ ($\epsilon = 3.5 \times 10^4$) and 254 mμ ($\epsilon = 1.3 \times 10^4$) for their feeding-factor in ethanol; moreover, the major absorption maximum of the methoxybipyrrolealdehyde shifts to 374 mμ in acid-ethanol and to 355 mμ in alkaline-ethanol (22).

Analytical paper chromatography As another means of characterizing our feeding-factor, its Rf values in various solvent systems were determined. Since the same chromatograms were used to determine the reaction of the feeding-factor to different spray reagents, solutions were streaked on 5-10 cm. sheets of paper using the method described for preparative chromatograms; usually, the ether extract from 200-400 ml. of cell-free broth was streaked on a 4.5-9.0 cm. line such that about 0.1-0.2 units of feeding-factor were streaked per cm. For comparison, similar chromatograms were streaked with 50 λ of a saturated solution of 4-methoxy-2,2'-

Figure 8. Absorption spectrum of the feeding-factor in methanol under acid, alkaline, and neutral conditions (neutral conc. one fourth others)


A
B
C
A ACIDIC
B ALKALINE
C NEUTRAL
ABSORBANCE
0.3
0.2
0.1
250
300
350
400
450
WAVELENGTH MU

bipyrrole-5-carboxaldehyde in dichloromethane. Chromatograms were developed for 20 hr. at 24° C. The results are shown in Table 1. It can be seen from the table that the Rf value of the feeding-factor is lower in the butanol-ammonia system than in the butanol-water system; thus, the compound probably contains an acidic functional group. In contrast, the Rf value of the methoxybipyrrolealdehyde is apparently unaffected by pH.

Table 1. Comparison of Rf's of orange mutant feeding-factor (FF) and 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC)

Solvent system	Average Rf FF[a]	Average Rf MBC
n-Butanol:water (1:1)	0.73	0.91
n-Butanol:1 N ammonia (1:1)	0.21	0.92
n-Propanol:n-butanol: 1 N ammonia (2:1:1)	0.39	0.93
n-Propanol:1 N ammonia (4:1)	0.32	0.83

[a]Applied as ether extract of cell-free broth.

Reactions with spray reagents When strips of paper chromatograms of the feeding-factor in crude ether extracts were sprayed with Ehrlich reagent a green spot developed at the Rf of the feeding-factor; the spot turned blue overnight. The same reaction to Ehrlich reagent is given by 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde, as reported by Santer (22)

and confirmed in this laboratory. When strips were sprayed with 1 per cent aqueous ferric chloride, one dark blue-grey spot at the Rf of the feeding-factor developed almost immediately. A spray consisting of 0.4 per cent 2,4-dinitrophenylhydrazine (2,4-DNP) in 2 N methanolic hydrochloric acid (9) gave no color. Identical positive and negative reactions were obtained with the methoxybipyrrolealdehyde. Ninhydrin-collidine spray (150 mg. of ninhydrin in 150 ml. of ethanol, 45 ml. of glacial acetic acid, and 6 ml. of collidine) gave no color even after heating the sprayed strip at 110° C for 10 min.

Treatment with diazomethane The spectral properties of the feeding-factor and its reaction to spray reagents suggested a compound similar in structure to 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde; however, the chromatography experiments showed that the feeding-factor has acidic properties absent in the methoxybipyrrolealdehyde. In view of the structure of norprodigiosin, it was considered that the feeding-factor might be the desmethyl anolog of the methoxybipyrrolealdehyde; an attempt was therefore made to convert the feeding-factor to a methoxy-anolog by treatment with diazomethane.

The ether extract from 800 ml. of cell-free broth was concentrated to about 50 ml. in vacuo at 30° C, transferred to a 500 ml. round-bottom flask, and cooled in an ice bath.

An ethereal solution of diazomethane, prepared from 2.0 g. of "Diazald", was distilled as prepared into the concentrated ether extract. The mixture was stirred with a magnetic stirrer at 0° C until the distillation was complete (usually 30 min.) and then allowed to stand at room temperature for 1 hr. After transfer to an evaporating dish and evaporation of excess diazomethane in a hood, the ether solution was concentrated *in vacuo* at 30° C.

In later experiments, the feeding-factor was eluted from preparative paper chromatograms with methanol, the methanol was removed *in vacuo*, and the residue was dissolved in ether for diazomethane treatment.

Properties of the diazomethane-treated feeding-factor

Paper chromatography When diazomethane-treated ether extract was streaked on paper in the usual fashion and chromatographed in n-butanol:1 N ammonia (1:1), no feeding-factor could be detected at Rf = 0.21, but a new feeding-factor spot was found at Rf = 0.83. Also, spraying with Ehrlich reagent, ferric chloride, and 2,4-DNP gave the same reactions as untreated feeding-factor and the methoxybipyrrolealdehyde at Rf = 0.83. The intensities of the spots of the new feeding-factor were at least as great as those produced by a corresponding amount of untreated feeding-factor, and in some cases were even greater; thus, the methylated product appears to be

more stable than the untreated feeding-factor.

The Rf values of the methylated feeding-factor and authentic 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde were compared in several different solvent systems. Three samples were spotted on the chromatograms: 2λ of a saturated dichloromethane solution of methoxybipyrrolealdehyde; about 0.7 units of methylated feeding-factor (one tenth of the diazomethane-treated ether extract from 800 ml. of broth); and finally a mixture of the two in the same quantities. The chromatograms were developed for 20 hr. at 24° C, dried, and assayed for feeding-factor on plates of the white mutant. The results are shown in Table 2.

Table 2. Comparison of Rf's of crude diazomethane-treated feeding-factor (DTFF) and 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC)

Solvent system	Average Rf MBC[a]	Average Rf DTFF[b]	Average Rf DTFF[b] + MBC
n-Butanol:water (1:1)	0.91	0.81	0.83
n-Butanol:1 N ammonia (1:1)	0.92	0.83	0.85
n-Propanol:n-butanol: 1 N ammonia (2:1:1)	0.93	0.88	0.88
n-Propanol:1 N ammonia (4:1)	0.83	0.77	0.80

[a]Values taken from Table 1.

[b]Applied as diazomethane-treated ether extract of cell-free broth.

It can be seen from Tables 1 and 2 that the Rf values of the diazomethane-treated feeding-factor are higher than those of the untreated feeding-factor in all the alkaline systems studied. Impurities with high Rf values tended to lower apparent Rf values of the diazomethane-treated feeding-factor in most of the systems, but mixtures of 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde and the diazomethane-treated ether extracts yielded only one feeding-factor spot.

Whenever crude ether extracts or ether solutions of the methanol eluate from preparative chromatograms of the feeding-factor were treated with diazomethane and taken to dryness in vacuo, a considerable amount of yellow oil was found in the residue. This material, apparently introduced during diazomethane treatment, also interfered with chromatography of the diazomethane-treated methanol eluate, reducing the Rf values of the methylated feeding-factor. Nevertheless, mixtures of the authentic methoxybipyrrolealdehyde and diazomethane-treated methanol eluate of the feeding-factor also yielded only one spot when chromatograms were assayed with the white mutant.

Counter-current distribution As another means of purifying the methylated feeding-factor and comparing it to the authentic methoxybipyrrolealdehyde, counter-current distribution was investigated. After distribution, the tubes of the apparatus were rinsed with absolute methanol, the volume

of solvent in each tube was brought to 5.0 ml. with methanol, and the absorbance at 363 mμ was read vs. a methanol blank. The distribution patterns obtained from 50-transfer runs of the authentic methoxybipyrrolealdehyde and diazomethane-treated methanol eluate in petroleum ether (b.p. 65-67° C): benzene:methanol:water (1:2:2:1) are shown in Figure 9. The contents of the tubes from the peak corresponding to K = 0.28 in the distribution of the methylated feeding-factor were pooled, concentrated to dryness in vacuo at 50° C, and re-distributed. The results from this run are also shown in Figure 9.

The contents of the tubes from the peak corresponding to K = 0.28 obtained in the second distribution of the methylated feeding-factor were concentrated to dryness and the residue was dissolved in a small volume of ether. Spots of the ether solution were chromatographed along with a reference spot of authentic methoxybipyrrolealdehyde. As shown in Table 3, the Rf of the methylated feeding-factor was essentially identical to that of the reference spot in the three solvent systems.

Counter-current distribution of untreated ether extract of cell-free broth in the petroleum ether-benzene-methanol-water system gave a peak corresponding to K = 0.20, but no feeding-factor could be recovered.

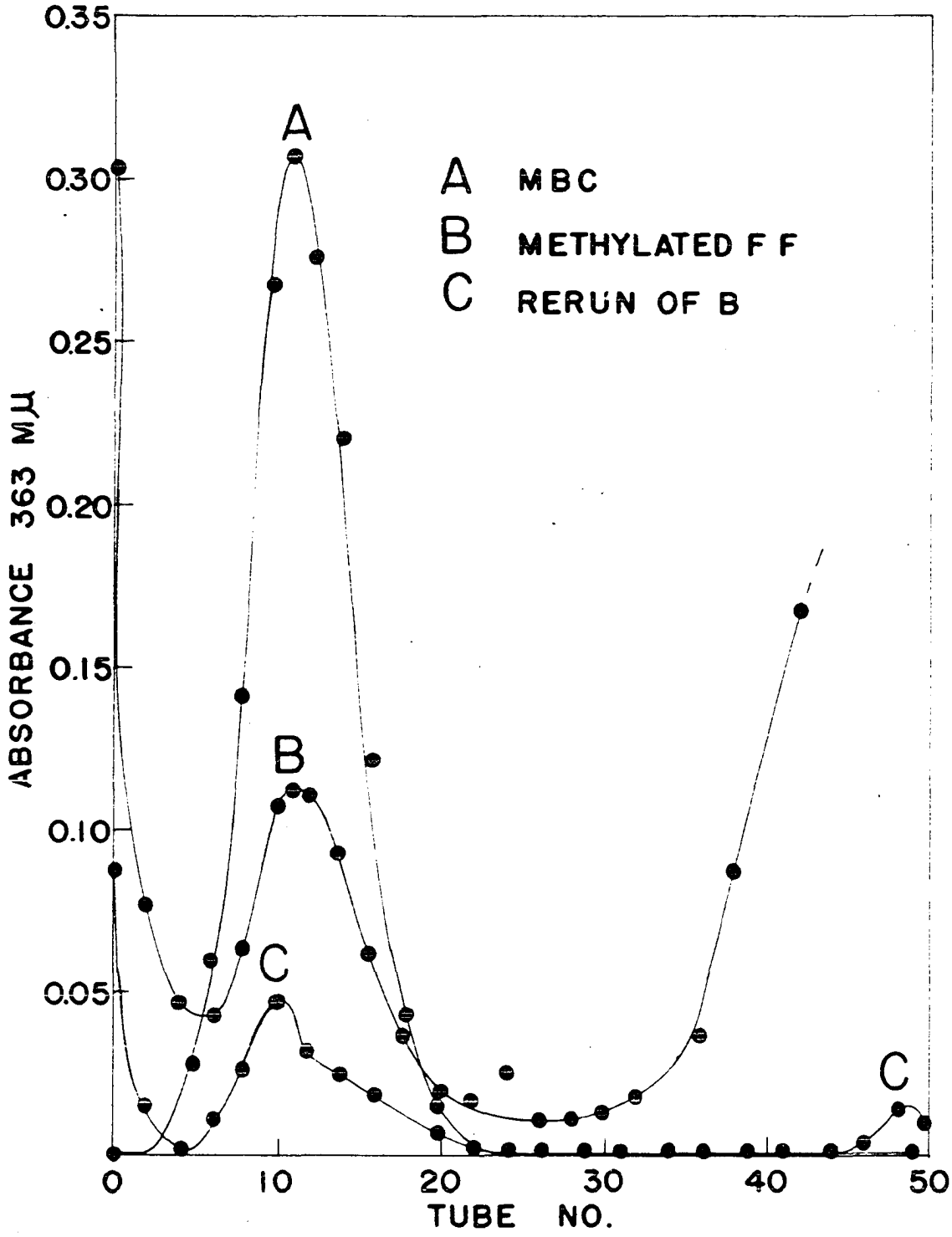


Figure 9. Counter-current distribution of 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC) and methylated feeding-factor in petroleum ether:benzene: methanol:water (1:2:2:1)

Table 3. Comparison of Rf's of purified diazomethane-treated feeding-factor (DTFF) and 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC)

Solvent system	Rf MBC	Rf DTFF
n-Butanol:1 N ammonia (1:1)	0.94	0.93
n-Propanol:n-butanol:1 N ammonia (2:1:1)	0.94	0.93
n-Propanol:1 N ammonia (4:1)	0.81	0.81

Feeding of the orange mutant If the orange mutant were blocked only in the methylation of 4-hydroxy-2,2'-bipyrrole-5-carboxaldehyde, then it should be able to synthesize prodigiosin if the methoxybipyrrolealdehyde were supplied to it. When paper strips containing either the authentic methoxybipyrrolealdehyde or diazomethane-treated ether extract of broth from cultures of the orange mutant were laid on the surface of 24 hr., 24° C Williams' agar cultures of the orange mutant, red spots developed within a few minutes.

Pyrroles Produced by Nima Strains

As mentioned in the previous section, the first approach in the investigation of the feeding-factor involved the assumption that the feeding-factor was a pyrrole derivative; thus, a number of observations were made concerning pyrroles

produced by the orange mutant. Also, some comparisons of pyrroles produced by the orange mutant, the white mutant, and the parent Nima strain were made.

Stability of pyrroles

Pyrroles in the broth of the orange mutant, like the feeding-factor, are quite unstable; the total measurable pyrrole dropped by about 50 per cent upon standing for 24 hr. in a refrigerator. After this initial rapid decline, the pyrrole level remained fairly constant when measured over a period of a week. In the experiments described below, only fresh cell-free broth was used.

Volatile pyrroles in the orange and white mutants

It was known that the biosynthesis of prodigiosin involves the coupling of 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde with the volatile 2-methyl-3-n-amylpyrrole (28). It has been shown in this investigation that both the orange mutant and the white mutant of the Nima strain can make red pigment when fed with the methoxybipyrrolealdehyde. Moreover, Santer (22) has reported that both the orange mutant and the white mutant of the Nima strain are capable of feeding the white mutant, strain 9-3-3, which is blocked in the biosynthesis of methylamylpyrrole (28). The following experiment was designed to show whether or not the Nima mutants produce

volatile pyrroles.

A Williams' agar plate was seeded with the orange mutant, OF, and another was seeded with the white mutant, WCF. After the plates had incubated for 24 hr. at 24° C, the covers were replaced with covers to which had been taped 8 x 1 1/2 cm. strips of Whatman No. 1 paper moistened with 5 drops of Ehrlich reagent and 5 drops of 2 N ethanolic hydrochloric acid. Red color was visible in the strips within a few minutes. After another 24 hr. of incubation at 24° C, the strip above the orange mutant had become quite pink and the strip above the white mutant had become deep purple. The strip above the white mutant was markedly more colored than the strip above the orange mutant, indicating that the white mutant gives off more volatile pyrrole. This would be expected, because the orange mutant undoubtedly incorporates some of its methylamylpyrrole into the orange pigment, norprodigiosin.

Effect of temperature on pyrrole production

Since pigment production in S. marcescens is blocked at 37° C (34), and since it had been shown in this investigation that production of the feeding-factor by the orange mutant is also blocked at 37° C, it was of interest to study the effect of temperature on the production of pyrroles.

When the experiment described above to test for volatile pyrroles was carried out at 37° C, little or no color

developed in the strips moistened with Ehrlich reagent. It would therefore appear that the biosynthesis of methylamylpyrrole is also blocked at 37° C.

Production of pyrroles in 24° C thin-layer cultures of the orange mutant has already been described (Figure 7). In one experiment the orange mutant was grown in thin-layer cultures for 48 hr. at 37° C; the cell-free broth from these cultures contained only 1 unit of pyrrole per liter as compared to 15 units per liter in corresponding 24° C cultures (both values corrected for base level of "pyrrole" in fresh Harned's broth).

Total pyrrole content in cultures of Nima strains

A comparison of the total pyrrole content (corrected for "pyrrole" in fresh Harned's broth) in cell-free broth from 48 hr., 24° C shake cultures of the Nima strains is given in Table 4. It can be seen from the table that the amounts of pyrroles produced in shake cultures are quite variable, with an average deviation of up to 85 per cent in the case of the white mutant; however, these data indicate that the orange mutant accumulates more pyrrole than the white mutant, and that both mutants accumulate more pyrrole than the wild-type strain.

The amount of pyrrole produced by the orange mutant in shake cultures was approximately seven times as high as the

Table 4. Total pyrroles in fresh cell-free broth from 48 hr., 24° C shake cultures of Nima strains

Strain	Average pyrrole content Units/L.	Number of determinations
Orange mutant, OF	110 ± 60[a]	24
White mutant, WCF	60 ± 50	15
Wild-type, Nima	2 ± 1	11

[a]Average deviation from the mean.

amount produced in thin-layer cultures; therefore, 48 hr., 24° C shake cultures were used to obtain cell-free broth for the experiments described below.

Extraction of pyrroles

When cell-free broth (pH ca. 7) from cultures of the orange mutant was extracted with three half-volume portions of diethyl ether, about 50 per cent of the pyrrole was removed from the broth; additional portions of ether gave no further extraction. Extraction of fresh orange mutant broth with n-butanol in the same proportions removed an average of 70 per cent of the pyrrole; extraction with ether followed by extraction with butanol removed up to 95 per cent. There was no significant difference in the amount extracted when the pH of the broth was adjusted to 2 or to 11.

In contrast to the results obtained for the orange mutant, an average of 95 per cent of the pyrrole in fresh cell-free broth from similar cultures of the white mutant could be extracted with ether.

Counter-current distribution

Samples of ether and n-butanol extracts were prepared for counter-current distribution by evaporating the solvents *in vacuo* at 30° C and 50° C, respectively. The distribution curve for a typical 20-transfer run in n-butanol:water (1:1) of n-butanol extract from 20 ml. of orange mutant broth is shown in Figure 10. The contents of some of the tubes from the peak corresponding to K = 1.1 from a similar run were concentrated to dryness *in vacuo* at 50° C, taken up in n-butanol:water (1:1) and redistributed. The results of this run are also shown in Figure 10. Recovery was about 50 per cent.

The distribution patterns of ether extract of orange mutant broth and n-butanol extract of ether-extracted broth are compared in Figure 11. It can be seen from these curves (Figures 10 and 11) that the butanol extract of orange mutant broth contains a pyrrole fraction which is not extracted into ether to any appreciable extent.

Counter-current distribution in n-butanol:water (1:1) of n-butanol extract of fresh cell-free broth from similar white

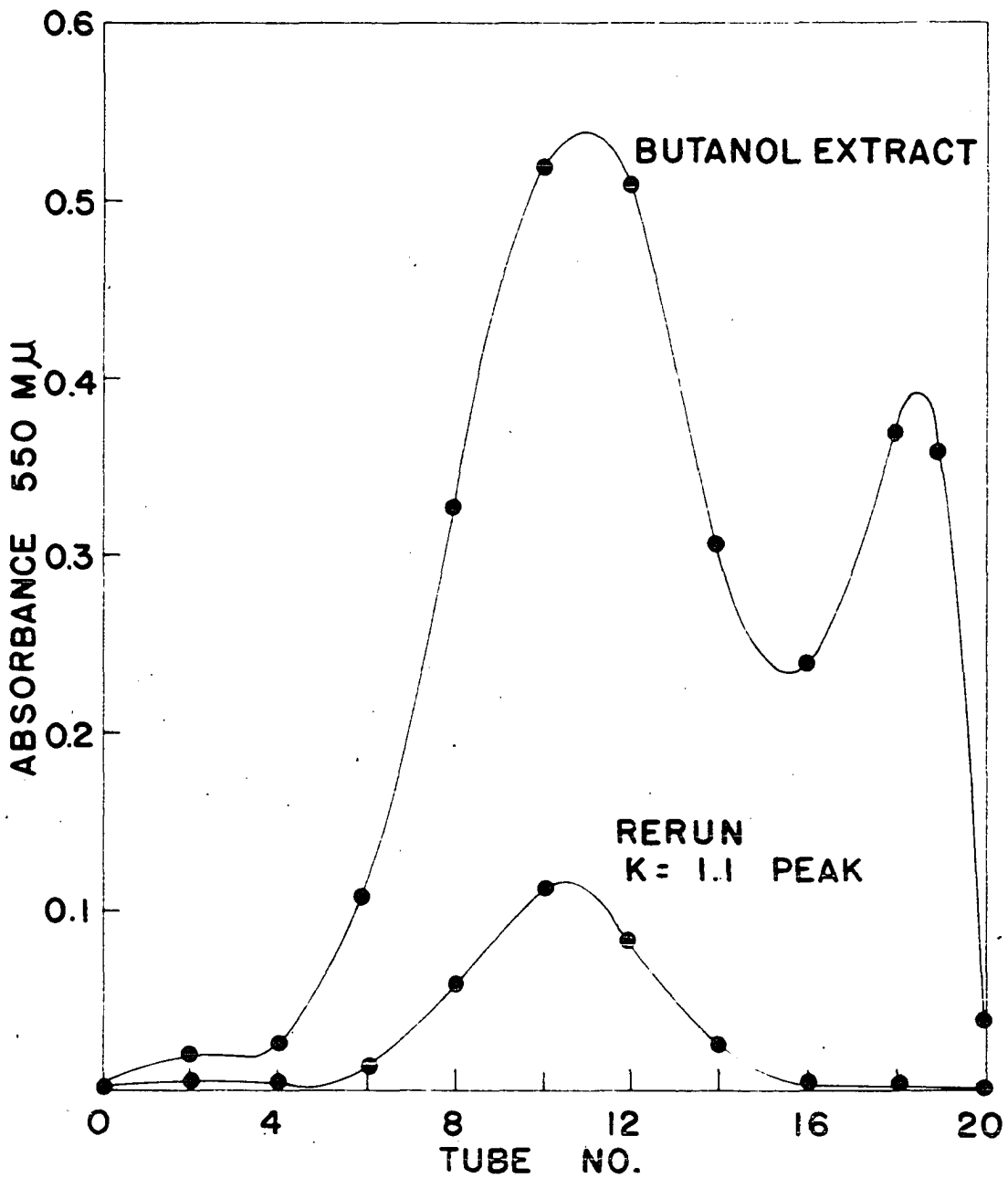


Figure 10. Counter-current distribution in n-butanol:water (1:1) of pyrroles in n-butanol extract of orange mutant broth

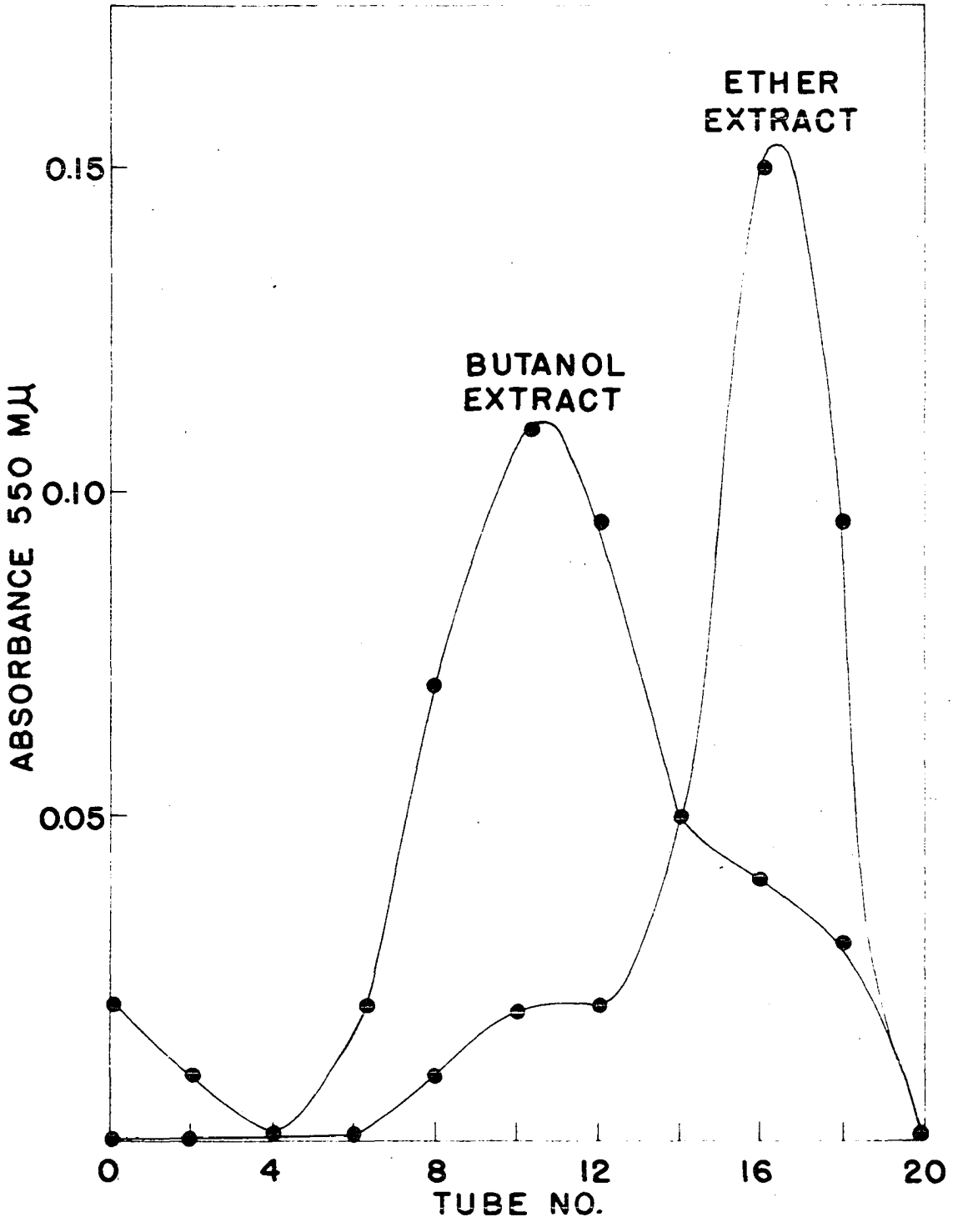


Figure 11. Counter-current distribution in n-butanol:water (1:1) of ether extract of orange mutant broth and of n-butanol extract of ether-extracted broth

mutant cultures gives only the fraction with a K of about 5.

Paper chromatography

When paper chromatograms of the ether extract of the orange mutant broth were sprayed with Ehrlich reagent, four to five blue or purple spots developed in addition to the green spot corresponding to the feeding-factor. Chromatograms of the n-butanol extract of ether-extracted orange mutant broth, and both fractions from counter-current distribution of butanol extract, none of which contained any detectable feeding-factor, also gave several Ehrlich-positive spots. These fractions have not been studied further.

DISCUSSION

The orange mutant, Serratia marcescens strain OF, and the white mutant, S. marcescens strain WCF, are both blocked in the biosynthesis of prodigiosin. The orange mutant has previously been shown to produce an abnormal pigment, norprodigiosin. The white mutant produces red pigment when the orange mutant and the white mutant are streaked adjacently on agar, when colorless metabolites can diffuse unidirectionally from the orange mutant to the white mutant, and when cell-free broth from cultures of the orange mutant is added to broth cultures of the white mutant. Prodigiosin can be isolated from mixed cultures of the two mutants in a yield comparable to the yield of prodigiosin isolated from the wild-type strain. Since the orange pigment does not diffuse into the medium, it follows that the colorless, diffusible feeding-factor produced by the orange mutant must be a precursor to prodigiosin.

Paper chromatography of ether extracts of cell-free broth of the orange mutant reveals only one compound capable of feeding the white mutant. This feeding-factor is similar to the known precursor to prodigiosin, 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC), in its spectral properties and its reactions with spray reagents, but it has acidic properties which are absent in MBC and it is less stable than MBC. Treatment of the feeding-factor with diazomethane

converts it in good yield to a new, more stable, feeding-factor which is identical to MBC in its reactions with spray reagents, in its behavior on paper chromatograms, and in counter-current distribution. Moreover, the diazomethane-treated feeding-factor, like MBC, enables the orange mutant to produce red pigment. Further comparisons of the untreated feeding-factor and the methylated feeding-factor with authentic MBC, by elemental analysis, infrared and n.m.r. spectra, etc., have not been made because of the limited amount of feeding-factor which can be isolated with the techniques now available; however, the evidence at hand is in good accordance with a 4-hydroxy-2,2'-bipyrrole-5-carboxaldehyde structure (structure V, Figure 12) for the precursor to prodigiosin accumulated by the orange mutant.

Isolation of large quantities of the feeding-factor for further studies of its physical and chemical properties presents a problem. Cell-free broth from shake cultures of the orange mutant contains about 27 units of feeding-factor per liter, based on the bio-assay. If 1 unit of feeding-factor is assumed to equal 1 μmole and the molecular weight of the feeding-factor is assumed to be 177 (see structure V), then 27 units per liter corresponds to about 5 mg. per liter. Thus, isolation of the feeding-factor in quantity would not appear to be an insurmountable problem as far as the amount present in fresh broth is concerned. However, the compound

is quite unstable, failing to survive any but the mildest of treatments. The ether extraction technique used to obtain small quantities for this investigation is inefficient, and the scaling-up of this process does not appear to be practical. The possibilities of extraction with other low-boiling solvents have not been exhausted, but it might be more practical in future work to try to find a stable derivative of the feeding-factor for the purpose of its isolation in large quantities (methylation, of course, being one possibility). Alternatively, it might be possible to stabilize the feeding-factor as a metal chelate.

The production of volatile pyrroles by the orange and the white mutants is in accordance with an accumulation of the volatile 2-methyl-3-n-amyl-pyrrole by these mutants. Cultures of the orange mutant produce less volatile pyrrole than cultures of the white mutant, probably because of the incorporation of some of its methylamylpyrrole into norprodigiosin.

Although the orange mutant produces less volatile pyrrole than the white mutant, the total amount of pyrrole accumulated in the orange mutant broth cultures is greater than the total amount accumulated in white mutant broth cultures. Since the feeding-factor gives a very weak Ehrlich test, the increased pyrrole level is probably not due to the feeding-factor *per se*, but is very likely due to pyrroles associated with the feeding-factor, either precursors to the feeding-factor or,

more likely, decomposition products of the feeding-factor. The results of the counter-current distribution experiments are consistent with such a relationship; butanol extracts of orange mutant broth, which contain no detectable feeding-factor, contain a pyrrole fraction which is not present in butanol extracts of white mutant broth; viz., the fraction with a K = 1.1 in the butanol-water solvent system.

In view of the structures of the feeding-factor and of norprodigiosin, it would seem reasonable that the feeding-factor should condense with methylamylpyrrole to form norprodigiosin in the orange mutant in a manner analogous to the condensation of MBC and methylamylpyrrole in the normal biosynthesis of prodigiosin. Measurements of total levels of pyrrole, feeding-factor, and orange pigment as a function of culture time (Figure 7) are in accordance with the incorporation of both pyrrole and feeding-factor into norprodigiosin.

Also, the yields of pigments obtained from pure cultures and mixed cultures of the Nima strains suggest that the feeding-factor is incorporated into norprodigiosin. Worthington has reported that the yield of prodigiosin obtained after diazomethane-treatment of norprodigiosin extracted from orange mutant cells corresponds favorably to the yield of prodigiosin extracted from wild-type cells (36). When the orange mutant is grown in mixed culture with the white mutant the yield of prodigiosin is comparable to the yield of prodigiosin from

similar cultures of the wild-type strain; moreover, countercurrent distribution of the fed-white pigment hydrochloride shows very little of any fraction which could be attributed to admixed orange pigment. It would appear, then, that feeding-factor is incorporated into the abnormal pigment, norprodigiosin, in pure cultures of the orange mutant, but that a competition exists in mixed cultures of the orange mutant and the white mutant, with a preferential incorporation of the feeding-factor into prodigiosin.

On the basis of the evidence discussed in the preceding paragraphs, and in view of the previously established reaction in prodigiosin biosynthesis (28), the scheme shown in Figure 12 is proposed for some of the final reactions in the biosynthesis of prodigiosin and norprodigiosin in the Nima strains. Both the orange mutant, OF, and the white mutant, WCF, can couple MBC (III) and methylamylpyrrole (IV) to form prodigiosin. The orange mutant cannot methylate 4-hydroxy-2,2'-bipyrrole-5-carboxaldehyde (V) and incorporates some of the accumulated compound into the abnormal pigment, norprodigiosin; very little, if any, norprodigiosin is formed in the wild-type strain. The white mutant is blocked in the biosynthesis of V, but can methylate V when it is fed by the orange mutant.

Studies by different investigators on the biosynthesis of prodigiosin have been carried out using different wild-type

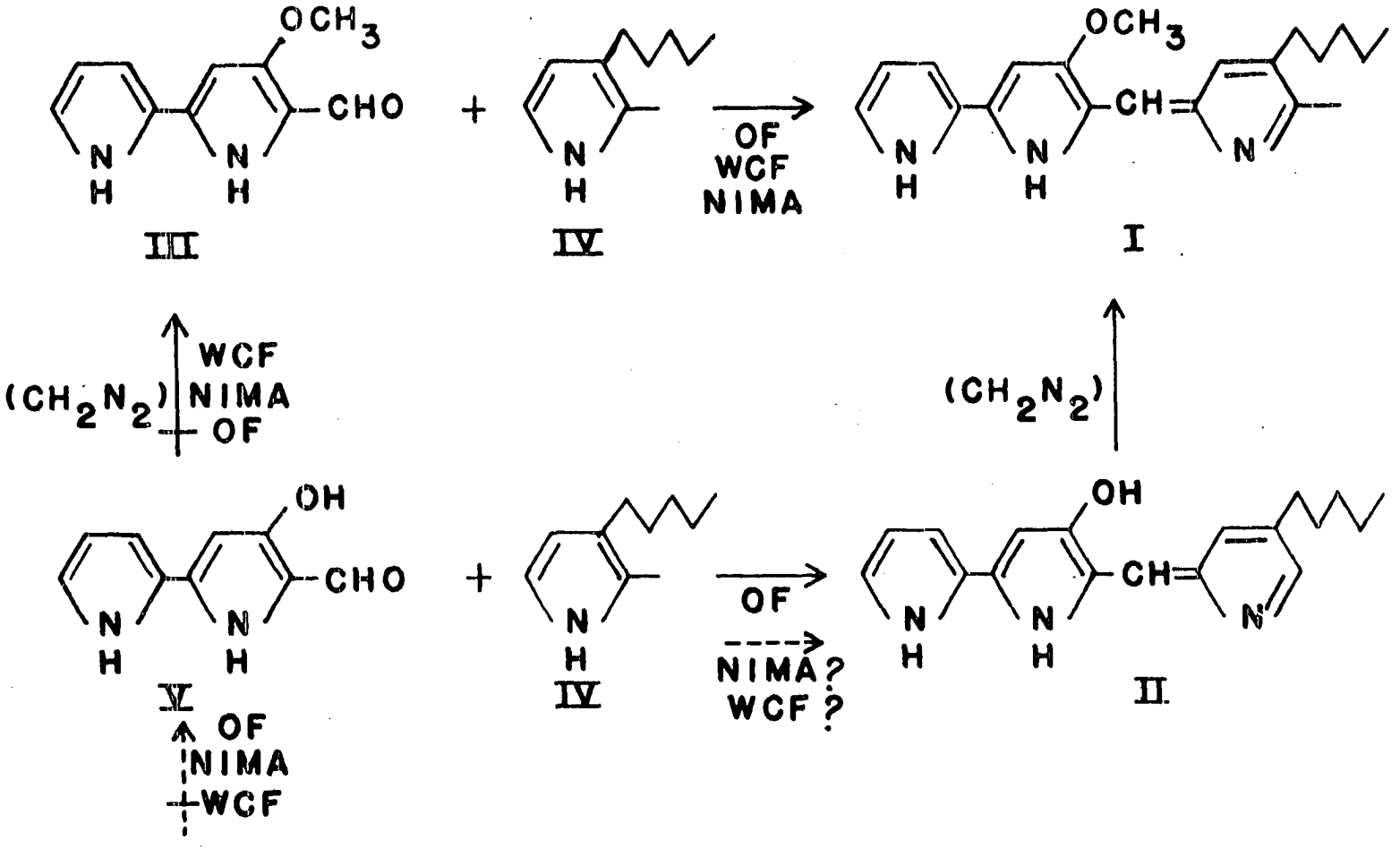


Figure 12. Proposed reactions in the biosynthesis of prodigiosin (I) and norprodigiosin (II) in Nima strains of Serratia marcescens

strains and mutants of different wild-type strains. The question then arises whether or not the biosynthesis of prodigiosin in the Nima strain is the same as the biosynthesis of prodigiosin in other wild-type strains. Santer (22) was able to demonstrate cross-feeding among the Nima, HY, and 274 strains of S. marcescens. The orange mutant, OF, of the Nima strain was identical in cross-feeding abilities to the orange mutants of the HY strain, strains O-3, 128, 131, and 133; and the white mutant, WCF, of the Nima strain was identical to the white mutant, W-1, of the HY strain. All of these orange mutants and white mutants were capable of feeding the white mutant, 9-3-3, of the HY strain, and all could be fed by 9-3-3; the orange mutants all fed the white mutants WCF and W-1, but none of the orange mutants were fed by WCF or W-1. Moreover, it has been found in this investigation that the precursor to prodigiosin produced by strain 9-3-3, MBC, is readily incorporated into red pigment by strains OF and WCF. This mutual cross-feeding among different strains indicates that the biosynthesis of prodigiosin proceeds in the same way in different wild-type strains, at least in the Nima and HY strains. There is also a strong possibility that the orange pigment produced by the orange mutants of other strains results from the formation of norprodigiosin from the hydroxybipyrrolealdehyde, V, as in the case of the orange mutant, OF. In this respect, Santer (22) has reported the presence

of an unstable, colorless feeding-factor in supernatants of broth cultures of the orange mutant, strain 128, which enables the white mutant, strain W-1, to produce red pigment.

The biosynthesis of prodigiosin or of norprodigiosin is known to be blocked in cultures grown at 37° C, even though growth is essentially normal at 37° C (34). Santer (22) has reported that the biosynthesis of MBC and the coupling of MBC with methylamylpyrrole are blocked when cultures of the HY strain are grown at 37° C; however, resting cell suspensions prepared from 30° C cultures could carry out the coupling reaction at 37° C, and the cells of the wild-type of the HY strain which had been grown at 37° were able to produce volatile pyrrole (presumably methylamylpyrrole) when tested at 30° C. In this investigation it was found that the biosynthesis of feeding-factor in the orange mutant and of volatile pyrrole in both the orange and the white mutants of the Nima strain were blocked when cultures were grown at 37° C, and that the total pyrrole level in orange mutant broth was greatly reduced in 37° C cultures. Thus, it can be seen that the elevation of the temperature from 30° C to 37° C has a general effect on the biosynthesis of prodigiosin, with the biosynthesis of the bipyrrole and of the monopyrrole precursors as well as their condensation to form prodigiosin blocked in some manner at the higher temperature. It might be possible to take advantage of this temperature effect in

studying prodigiosin biosynthesis; perhaps some other precursors to prodigiosin are accumulated when cultures are grown at 37° C.

With the establishment of the nature of the blocks in the biosynthesis of prodigiosin in the orange mutant, OF, and the white mutant, WCF, it becomes apparent that these mutants might be used for further elucidation of the pathways of biosynthesis of prodigiosin. The white mutant, blocked in the biosynthesis of the bipyrrole precursor to prodigiosin, may accumulate a precursor to the bipyrrole which could be isolated and identified, unless the accumulated precursor is a simple compound which could enter a metabolic pool. The orange mutant, blocked in the methylation of the hydroxybipyrrolealdehyde, might be useful in elucidating the nature of the methylation reaction.

It is known from labeling experiments (13, 15) that L-proline, glycine, and acetate are incorporated into prodigiosin, and that glycine is incorporated into the bipyrrole precursor (23). The evidence in the literature is not in accordance with the biosynthesis of the pyrrole rings in prodigiosin from the precursors to porphyrins, 5-aminolevulinic acid and porphobilinogen (15); experiments with labeled glycine and acetate (13) suggest that some of the initial reactions in the biosynthesis of the prodigiosin pyrroles might be analogous to the biosynthesis of 5-aminolevulinic acid from

glycine and succinate. It would be of interest to determine more specifically the nature of incorporation of proline, glycine, and acetate into prodigiosin; since the orange mutant and the white mutant accumulate the bipyrrole and monopyrrole precursors, the relative extents of incorporation of labeled proline, glycine, and acetate into the bipyrrole and monopyrrole precursors to prodigiosin could be determined using these two mutants. In view of the instability of the bipyrrole accumulated by the orange mutant, however, it might be better to carry out specific labeling studies on the bipyrroles using a mutant which accumulates the more stable methoxybipyrrole.

Other than L-proline, glycine, and acetate, the only intermediates in prodigiosin biosynthesis described in the literature are 2-methyl-3-n-amyl-pyrrole and 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (28). In the investigation reported here, evidence has been obtained for the existence of another intermediate in prodigiosin (and norprodigiosin) biosynthesis: 4-hydroxy-2,2'-bipyrrole-5-carboxaldehyde.

SUMMARY

1. Experiments were conducted on an orange mutant, Serratia marcescens strain OF, and a white mutant, S. marcescens strain WCF, which were obtained by irradiation of the wild-type strain Nima (34) and which are both blocked in the biosynthesis of prodigiosin, the blood-red pigment normally produced by S. marcescens.
2. Strain OF, which produces the abnormal orange pigment, norprodigiosin (36), was shown to accumulate in the culture medium an unstable, colorless, diffusible feeding-factor which enables strain WCF to produce red pigment.
3. Prodigiosin was isolated by established methods from mixed cultures of strains OF and WCF in a yield comparable to the yield of prodigiosin isolated from cultures of strain Nima; norprodigiosin was apparently absent in the mixed cultures.
4. Paper chromatography of diethyl ether extracts of cell-free broth from cultures of strain OF revealed a single feeding-factor spot by its red coloration when chromatograms were assayed on the surface of an agar culture of strain WCF.
5. The feeding-factor, purified by paper chromatography, was found to be similar to the known precursor (28) to prodigiosin, 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC), in spectral properties and in reactions with spray

reagents, but was much less stable than MBC and exhibited acidic properties on paper chromatograms absent in MBC.

6. Paper chromatography of diazomethane-treated ether extracts of cell-free OF broth or diazomethane-treated eluate from chromatograms of the feeding-factor revealed none of the original feeding-factor, but instead, a new, more stable feeding-factor which was identical to authentic MBC in reactions with spray reagents, in behavior on paper chromatograms, and in counter-current distribution. Authentic MBC and diazomethane-treated feeding-factor both enabled strain OF to produce red pigment.
7. Strains OF and WCF were each shown to produce volatile pyrrole, presumably the known volatile precursor to prodigiosin, 2-methyl-3-n-amyl-pyrrole (28), but strain OF produced less volatile pyrrole than strain WCF; biosynthesis of volatile pyrrole by strains OF and WCF, and the biosynthesis of feeding-factor and pigment by strain OF, were both shown to be blocked in cultures grown at 37° C.
8. Measurements of total levels of pyrroles, feeding-factor, and orange pigment as a function of culture time in broth cultures of strain OF were in accordance with the incorporation of both pyrroles and feeding-factor into orange pigment; n-butanol extracts of cell-free broth from cultures of strain OF contained a pyrrole fraction which was

shown by counter-current distribution to be absent in similar extracts from cultures of strain WCF.

9. Therefore, it is proposed that the feeding-factor accumulated by strain OF is 4-hydroxy-2,2'-bipyrrole-5-carboxaldehyde, which is methylated and then combined with methylamylpyrrole in the normal biosynthesis of prodigiosin in strains Nima and WCF, and which is combined directly with methylamylpyrrole to form the abnormal pigment, norprodigiosin, in strain OF.

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